



PHD

ADPR-transferase in T. brucei

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ADPR-transferase in T.brucei

A thesis submitted in fulfilment of
the requirements for the degree of
Doctor of Philosophy at the
University of Bath

by

Mahnaz Alsharif

1988

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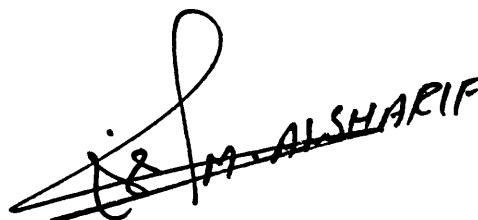
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A handwritten signature in black ink, appearing to read 'I. S. M. ALSHARIF'. The signature is stylized with a large loop at the beginning and is written over a horizontal line.

Dedicated to all the people whose labour
paves the way to the better
understanding of the world we live in

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ABBREVIATIONS

3AAB	3-Acetamidobenzamide
3AABA	3-Acetamidobenzoic acid
3AB	3-Aminobenzamide
3ABA	3-Aminobenzoic acid
Ad	Adenine
ADH	Alcohol dehydrogenase
ADP	Adenosine diphosphate
ADPR	Adenosine diphosphate ribose
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BHK Cells	Baby Hamster Kidney Cells
cpm	Counts per minute
CsCl	Caesium Chloride
cAMP	Cyclic adenosine monophosphate
DEAE-Cellulose	Diethylamine ethyl cellulose
DMS	Dimethylsulphate
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
EGTA	Ethyleneglycol-bis-(aminoethyl ether)- N-N -tetra-acetic acid
EtoH	Ethanol
Glu	Glutamine
Glu(NH ₂)	Glutamate
HEPES	N-2-Hydroxyethyl-piperazine-N'-2- -ethane sulphonic acid
K-DNA	Kinetoplast-DNA

Ki	Inhibition-constant
MTT	Thiazolyl blue
Na	Nicotinic acid
NaAD	Nicotinate adenine dinucleotide
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced form of nicotinamide adenine dinucleotide
NaMN	Nicotinic acid mononucleotide
NM	Nicotinamide
NMN	Nicotinamide mononucleotide
PBS	Phosphate buffered saline
PMS	Phenazine methosulphate
PPO	2,5-Diphenyl-oxazole
PR-AMP	2'-(5"-Phosphoribosyl)-5'AMP
Rib	Ribose
RNA	Ribonucleic acid
SHAM	Salicylhydroxamic acid
TCA	Trichloroacetic acid
Tris	2-amino-2-hydroxymethyl propane-1-3-diol
tRNA	Transfer RNA
U.V	Ultra violet
VSG	Variant surface glycoprotein

ABSTRACT

Nuclear adenosine diphosphoribosyl transferase (ADPR-transferase) is present in all nucleated cells in animals, plants and lower organisms studied so far, with the exception of a number of terminally differentiated cells in which the nuclei have ceased transcription. This enzyme which catalyses the formation of mono, oligo and poly(ADP-ribose)-modified chromatin proteins from NAD, is entirely dependent on DNA containing strand-breaks for its activity. It has been demonstrated that nuclear ADPR-transferase activity is required for efficient DNA excision repair and a number of other cellular processes which may involve DNA strand-break formation and rejoining, such as cellular differentiation, mitogenic activation of quiescent lymphocytes, sister chromatid exchange, antigenic variation in T.brucei and the stable expression of transfected DNA in mammalian host cells.

The work described here has demonstrated the presence of ADPR-transferase activity in bloodstream forms of T.brucei and its activation by DNA strand-breaks, either by exposure to the monofunctional methylating agent, dimethyl sulphate (DMS), or ionising radiation (γ -radiation). Inhibitors, but not the non-inhibitory acid analogues, partially block the DNA damaging agent induced lowering of cellular NAD level. These inhibitors also potentiate the cytotoxicity of both DMS treatment and γ -irradiation. It is also demonstrated that in vitro differentiation of bloodstream forms of T.brucei to procyclic forms can be partially blocked by inhibitors of ADPR-transferase. However the inhibition of this enzyme did not effect the rate of proliferation.

Chapter 1

Introduction to ADP-ribosylation

1.1 Biosynthesis of NAD

Since early 1950's a number of studies have shown that NAD (Fig. 1.1) as well as being a coenzyme in many biological oxidation-reduction reactions, also participates in other important biological processes and is the unique substrate for the biosynthesis of mono, oligo and poly(ADP-ribose) in ADP-ribosylation reactions.

Generally the cellular synthesis of NAD may be divided into cytoplasmic steps and nuclear steps and is essentially a cyclic pathway which occurs in three quite different ways (Fig. 1.2).

A - The cytoplasmic conversion of amino acid tryptophan to nicotinic acid mononucleotide (NaMN) through 3-hydroxyanthranilic acid and quinolinate. This pathway was found to be present in the liver (Nishizuka & Hayaishi, 1963 and Nakamura et al., 1963) and mammalian kidneys only. It has been shown that several mammalian cell lines are unable to synthesise nicotinamide from tryptophan (Hillyard et al., 1973 and Jacobson et al., 1979).

B - Condensation of nicotinic acid with phosphoribosyl pyrophosphate (PRPP) to form nicotinic acid mononucleotids (NaMN) in avian liver (Preiss & Handler, 1958a,b), in avian kidney (Sarma, 1961) as well as in chicken erythrocytes (Lin & Henderson, 1972). It has been observed that many tissues are unable to convert nicotinic acid to NaMN directly (Collin & Chaykin, 1972 and Lin & Henderson, 1972). This condensation reaction is catalysed by the enzyme nicotinate phosphoribosyl transferase (NaPRT). NaMN produced in this way migrates to the nucleus where it is condensed with ATP to give nicotinate adenine dinucleotide (NaAD). The enzyme catalysing this step is nicotinate mononucleotide adenylyl-transferase. The synthesis of NAD from nicotinic acid represents an important pathway especially in the liver.

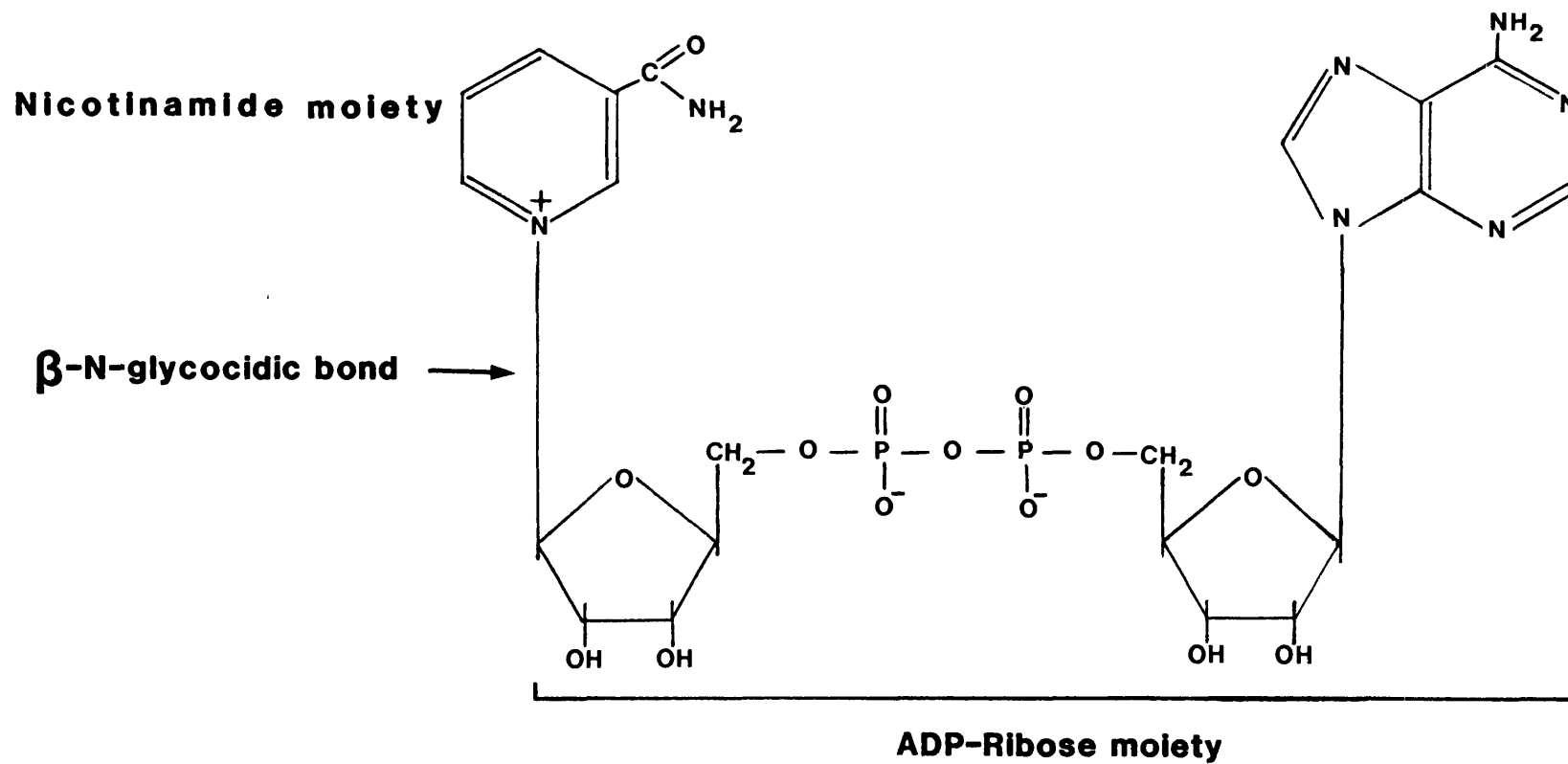


Fig. 1.1 STRUCTURE OF NAD

Fig. 1.2 NAD Metabolising Enzymes in Nucleated Cells

A Cytoplasmic Enzymes

1. Quinolinate synthetic enzyme
2. Quinolinate phosphoribosyl-transferase
3. Nicotinate phosphoribosyl-transferase (NaPRT)
4. Nicotinate deaminase
5. Nicotinamide phosphoribosyl-transferase (NmPRT)
6. NAD synthetase
7. NAD glycohydrolase

B Nuclear Enzymes

1. Nicotinate mononucleotide adenylyl-transferase
2. NAD-pyrophosphorylase
3. ADPR-transferase
4. ADPR-glycohydrolase
5. NAD phosphokinase

Np = Nuclear protein

C - In this way nicotinamide is the direct precursor of the NMN. The reaction occurs in the cytoplasm with the condensation of nicotinamide with phosphoribosyl pyrophosphate (PRPP) to form nicotinamide mononucleotide (NMN), catalysed by nicotinamide phosphoribosyl-transferase (NmPRT), NMN then diffuses into the nucleus and there the enzyme NAD-pyrophosphorylase catalyses the condensation of NMN and ATP to form NAD with the release of inorganic pyrophosphate. This pathway occurs in rat mammary gland (Greenbaum & Pinder, 1968), mouse tissues (Streffer & Benes, 1971 and Collins & Chaykin, 1972), and cellular levels (Grunicke et al., 1966; Hillyard et al., 1973 and Jacobson, 1979). The NAD synthesised in the nucleus is then used by the nuclear enzyme ADPR-transferase for biosynthesis of poly(ADP-ribose).

The study of NAD metabolism in eukaryotic cells has taken on an increased significance in recent years. This interest concerns the transfer of ADP-ribose residues from NAD to cellular proteins or to the adenine ribose of another ADP-ribose molecule, a process called ADP-ribosylation. Reichsteiner et al. (1976 a,b) have shown that the half life of NAD in human HeLa cells is about 1 hour, and that only about 5% of the NAD synthesised in the cell is required for redox reactions. The other 95% is hydrolysed in the nucleus, almost certainly catalysed by ADPR-transferase (Fig. 1.2).

1.2 ADPR-transferase

Both in terms of function and cellular location, there are at least two classes of ADPR-transferase enzymes: nuclear(ADPR-transferase), and non-nuclear(ADPR-transferase).

1.2.1 Nuclear(ADPR-transferase)

Nuclear(ADPR-transferase) is a chromatin-bound enzyme. The presence of this nuclear enzyme has been reported in most nucleated cells in animals, plants and lower eukaryotic cells studied so far. The exceptions being that a number of terminally differentiated cells in which the nuclei have ceased transcription eg. mature erythrocytes (Nishizuka et al., 1967), mature granulocytes (Ikai et al., 1980a,b; Ikai et al., 1981) and intestinal epithelial cells (Porteous & Pearson, 1982). The presence of this enzyme activity has also been reported in protozoan parasites Plasmodium yoelii (Okolie & Onyezili, 1983), Trypanosoma cruzi (Williams, 1983b) and Trypanosoma brucei (Farzaneh et al., 1985; Alsharif et al., 1986a,b). The involvement of ADPR-transferase activity in trypanosomes will be discussed further in chapter 2. Nuclear(ADPR-transferase) catalyses the postsynthetic modification of nuclear proteins by the covalent attachment of the (ADP-ribose) moiety of NAD with the concomitant release of nicotinamide and hydrogen ions plus mono, oligo and poly(ADP-ribose) (Fig. 1.3). (ADP-ribose)_n-modification of proteins involves at least three steps:

- 1- The first reaction is to transfer the ADP-ribose portion of NAD to the protein acceptor, to form mono-ADP-ribosyl protein (Fig. 1.3A).
- 2- The next is the transfer of a second ADP-ribose portion of NAD to the mono-ADP-ribosyl protein (Fig. 1.3B). In this reaction the second ADP-ribosyl unit is linked by a (1"-2') glycosidic bond to the adenine ribose of the preceeding ADP-ribose (Miwa et al., 1979, Juarez-Salinas et al., 1982). This ADPR addition reaction may be repeated, generating a

homopolymer of poly(ADP-ribose), covalently linked to a chromatin protein (Fig. 1.3B).

3- A further reaction involves the formation of branched poly(ADP-ribose). This branching structure has been shown to be present in polymer synthesised in vitro (Tanaka et al., 1978) and in vivo (Miwa et al., 1979, 1981). The branching chains produced by the formation of a second α -(1"-2') glycosidic bond between a ribose molecule inside the polymer and another ADP-ribose molecule (Miwa et al., 1979; Miwa et al., 1981) (Fig 1.3C). The polymer which may be up to 65 units long is soluble in water and in alkaline solutions, but is precipitated by acids (Fujimura & Sugimura, 1971, and Sugimura, 1973).

The enzyme has an absolute requirement for DNA strand-breaks (Benjamin & Gill, 1978, 1980a; Yoshihara et al., 1982; Cohen et al., 1982; Ueda et al., 1982; Cleaver et al., 1983; Ohgushi et al., 1980; Cohen & Berger, 1981; Yoshihara et al., 1980; Berger et al., 1978a, 1979, 1980; Ito et al., 1979; Haldorsson et al.; 1978; Smulson et al., 1977 and Miller 1975 a,b). Also it has been shown that ADPR-transferase requires Mg^{++} and thiol reagents such as DTT (dithiothreitol) for maximal activity (Yoshihara et al., 1978; Nierdergang 1979, and Carter & Berger 1981).

Purification of nuclear ADPR-transferase has been attempted by several workers (Yoshihara, 1972; Ueda et al., 1975; Okayama et al., 1977; Tsopanakis et al., 1976, 1978; Shizuta et al., 1985, 1986; Burtscher et al., 1986 and Suzuki et al., 1987). The most recent estimates of the molecular weight of nuclear ADPR-transferase is in the range of 115000-120000 (See table 1.1). This enzyme is a

Pa=Protein acceptor

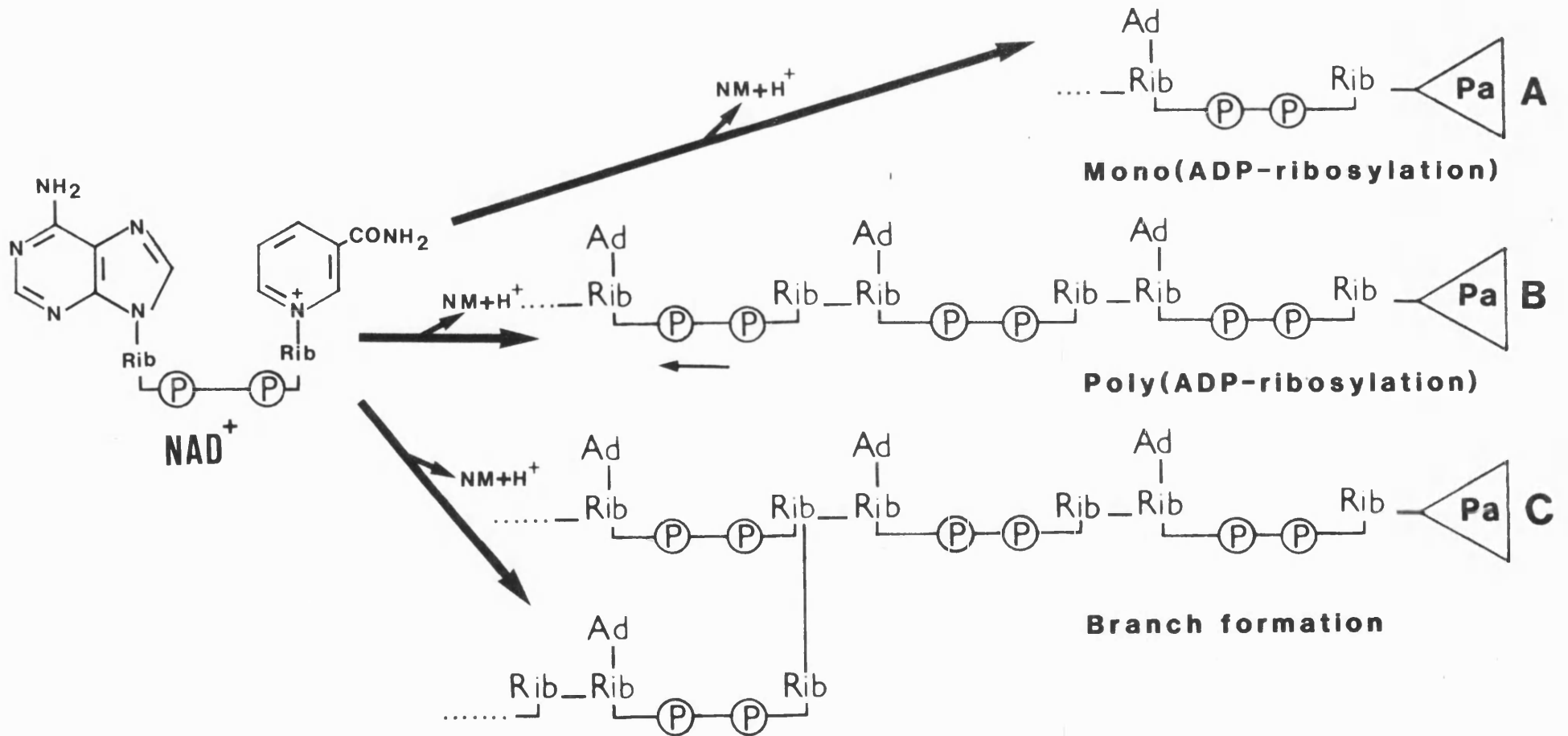


Fig. 1.3 ADP-ribosylation

<u>TISSUE/CELLS</u>	<u>M.Wt.x10³</u>	<u>pH OPT.</u>	<u>TEMP^oC</u>	<u>REFERENCE</u>	<u>DNA DEP./INDEP</u>
Calf thymus	120	8.0	35	Hayaishi et al., 1979	indep.
	150	-	30	Mandel et al., 1977	dep.
				Gill 1972	-
Bovine thymus	130	8.5	25	Yoshihara et al., 1978	dep.
Pig thymus	63	8.0	25	Tsopanakis et al., 1978	dep.
Ehrlich ascities tumor cells	130	8.0	25	Houtland 1978	dep.
Hela cells	125	8.0	25	Jump et al., 1980	dep.
	112	-	-	-	-
Rat liver nuclei	50	-	+	Hayaishi 1977	-
	150	-	-	Yoshihara & Koide 1973	-
	100	-	-	-	-

Table 1.1 continued:

<u>TISSUE/CELLS</u>	<u>M.Wt. x10³</u>	<u>pH OPT.</u>	<u>TEMP^oC</u>	<u>REFERENCE</u>	<u>DNA DEP./Indep</u>
Beef, rat, pig, lamb thymus, chicken	130	-	-	Jongstra - Bilen 1981 Petzold 1981	- -
Tonsil	-	-	-	Carter & Berger 1982	-
Human placenta	115	9.0	25	Burtscher et al., 1986	dep.
Calf thymus gland, mouse testis, Human placenta	120	-	-	Shizuta et al., 1985-86	dep.

globular protein (Ito et al., 1979) with an iso-electric point of 9.4 (Kristensen and Holtlund, 1978) or 9.8 (Ito et al., 1979), and the N-terminus appears to be blocked (Tsopanakis et al., 1978; Ito et al., 1979). The purified enzyme catalyses the ADP-ribosylation of a range of nuclear proteins including histones (Yoshihara et al., 1980 and Mandel et al., 1980), as well as the auto-modification of the enzyme molecule itself (Yoshihara et al., 1977; Ueda et al., 1979; Ogata et al., 1981; Hayaishi et al., 1981; Agemori et al., 1982; Kawaichi et al., 1981 and Zahradka & Ebisuzali, 1982). The ability of the enzyme to modify itself increases with increasing ionic strength (Ferro & Oliver 1982). ADPR-transferase, purified from calf thymus gland, mouse testis, and human placenta, was found to be cleaved by limited proteolysis into 3 different domains (Shizuta et al., 1985 & 1986). The first domain (mol wt = 46,000) contained the site for DNA binding, the second domain (mol. wt = 22,000) contained the poly(ADP-ribose)-accepting (auto-modification) site, and the third domain (mol. wt. = 54,000) contained the NAD (substrate)-binding site. The amino acid components of enzyme from all three different sources were also very similar to each other (Shizuta et al., 1985). Burzio et al., 1981, showed the presence of a high ADPR-transferase activity in isolated rat testis mitochondria. Inhibition studies showed that like the nuclear enzyme, the rat testis mitochondrial enzyme can be inhibited completely by inhibitors of the nuclear ADPR-transferase activity such as nicotinamide, theophylline and thymidine (Kun et al., 1975). The presence of ADPR-transferase in nucleoli has also been reported (Kawashima and Izawa, 1981).

Antibodies developed against ADPR-transferase (Rochett et al., 1980; Malik et al., 1982 and Lamarre et al., 1986) have shown great differences in their reaction with ADPR-transferase derived from

different sources. This suggests some species variation in the enzyme structure. The recent molecular cloning of the ADPR-transferase gene and the subsequent analysis of its nucleic acid sequence stands to provide the most direct evidence on the degree of conservation of ADPR-transferase structure in different species. The probes have been used to investigate mRNA expression in the HeLa cell cycle (Alkhatib et al., 1987) and during HL-60 cell differentiation (Suzuki et al., 1987).

1.2.2 Non-nuclear(ADPR-transferase)

Non-nuclear mono(ADP-ribose) modification of proteins has been observed in both prokaryotes and eukaryotes. The characteristics and properties of the eukaryotic mono(ADPR-transferase) is briefly examined below. Despite the discovery of numerous examples of mono(ADPR-transferase) activities in prokaryotes, until 1978 a similar activity had not been isolated from eukaryotic cells. In 1978 Moss and Vaughan described a cytosolic mono(ADP-transferase) activity in turkey erythrocytes and subsequently purified this enzyme to homogeneity (Moss and Vaughan, 1978 & 1980). The enzyme found in turkey erythrocytes modified arginine and its derivatives, and was capable of activating the adenylate cyclase of rat brain in the presence of NAD. This enzyme existed in two different forms, protomeric (fully active) and oligomeric (relatively inactive). Activation was promoted by chaotropic salts, histones (Moss & Vaughan, 1982), and non-ionic detergents (Ueda et al., 1982). Although NAD was clearly the preferred substrate, the enzyme could use NADP (Moss & Vaughan, 1982). The enzyme activity was inhibited by nicotinamide, thymidine, and theophylline (Moss et al., 1980), as is poly(ADPR-transferase). Furthermore, membrane fractions of

various mammalian cells were found to possess endogenous ADPR-transferases (Beckner & Beckner, 1981; Walaas et al., 1981; Vitti et al., 1982; Lester et al., 1982).

The finding of these arginine-specific ADPR-transferases in vertebrates carried two major implications, one was the possible roles in signal transmission; and the other was concerned with heterogeneity of ADP-ribosyl protein bonds. The finding of stimulation of rat liver adenylate cyclase by the turkey erythrocyte enzyme (Moss & Vaughan, 1978), the stimulation of ADP-ribosylation by specific hormones (Vitti et al., 1982), and ADP-ribosylation of the α subunit of N_G , by endogenous enzymes and cholera toxin (Beckner & Beckner, 1981; DeWolf et al., 1981; Vitti et al., 1982) all support the view that the vertebrate enzymes might function as physiological counterparts of cholera toxin (Cassel & Pfeuffer, 1978 and Gill & Meren, 1978) or E. coli enterotoxin LT (Hayaishi & Ueda, 1982).

1.3 ADP-ribosylation of Cellular Proteins

ADP-ribosylation is a protein modification process which occurs widely in nature and has considerable importance in the regulation of enzyme activity. As mentioned earlier, NAD is used in the process not as a cofactor as in biological redox reactions, but as a substrate in a molecular mechanism for modulating the activity of a wide variety of cellular proteins (Hayaishi & Ueda 1982; Gaal & Pearson, 1985 and Ueda & Hayaishi, 1985). The reactions are catalysed by the enzymes nuclear and non-nuclear(ADPR-transferase).

In eukaryotes, such reactions take place both inside and outside the cell nucleus. Poly(ADP-ribosylation) appears to be confined to the eukaryotic nucleus; mono(ADP-ribosylation) occurs throughout the

eukaryotic cell: in the nucleus, cytoplasm, mitochondria and using membrane proteins as substrates.

Both poly and mono(ADP-ribosylation) has been the subject of a number of extensive reviews (Sugimura, 1973; Hilz & Stone, 1976; Hayaishi & Ueda, 1977 and Purnell et al., 1980). For more recent reviews see (Shall, 1984; Althaus et al., 1985; Ueda & Hayaishi, 1985 and Gaal & Pearson, 1986).

The purpose of this introduction is therefore not an extensive review of the field, but rather a summary relating the diverse areas of ADP-ribosylation, DNA repair, cellular differentiation and trypanosomiasis.

1.3.1 Nuclear(ADP-ribosylation) and its Biological Significance

The nuclei of all cells studied thus far contain an enzymic system capable of post-synthetic modification of nuclear proteins by the covalent attachment of the (ADP-ribose) moiety of NAD (Hayaishi & Ueda, 1982 and Ueda & Hayaishi, 1985). Although "nuclear" is often used synonymously with "poly" when describing ADP-ribosylation, this is incorrect. There is a nuclear mono(ADPR-transferase) (Tanigawa et al., 1984), however it does not represent the majority of ADPR-transferase activity in the nucleus. Adamietz et al. (1984) estimate that less than 5% of the total cellular mono-ADP-ribosylated proteins are located in the nucleus. The enzyme appears to be specific for arginine residues. The acceptors of ADP-ribose molecules include both histones and non-histone nuclear proteins. As long ago as 1968, Nishizuka et al., suggested that histones H1, H2a, H2b and H3 of rat liver could all be covalently linked to poly(ADP-ribose). ADP-ribosylation of histones has since been reported not only in

mammalian nuclei (Smith & Stocken 1973 and Ueda et al., 1975) but in the sea urchin, Echinus esculentus (Ord & Stocken 1977) and in trout testis (Wong et al., 1977).

The covalent linkage of ADP-ribose to proteins was first postulated by Nishizuka et al. (1968 & 1969). This was later proved by the demonstration that the ADP-ribose linkage to histones was preserved during centrifugation in a CsCl density gradient containing guanidinium hydrochloride (Otake et al., 1969). There are at least two different types of bonds between ADP-ribose and acceptor proteins. One type being sensitive to both alkali and neutral hydroxylamine, the other being alkali labile but resistant to hydroxylamine (Adamietz & Hilz 1975, 1976).

The initial observation which later led to the discovery of the poly(ADP-ribose), a homopolymer of adenosine diphosphoribose, was made in the laboratory of Chambon in Strasbourg in 1963. Chambon and co-workers reported that in the presence of nicotinamide mononucleotide (NMN), hen liver nuclei could incorporate [adenine ¹⁴C]-ATP into an acid-insoluble material (Chambon et al, 1963). The incorporation of [¹⁴C]-ATP into acid-insoluble material was shown to be mediated through the formation of NAD from ATP and NMN by NAD pyrophosphorylase (Chambon et al., 1966; Fujimura et al., 1967 and Sugimura et al., 1967). Thus in three different laboratories it was shown that NAD was the true substrate for the nuclear enzyme ADPR-transferase. The product could be cleaved by snake-venom phosphodiesterase to a nucleotide containing two ribose units and two phosphate residues per adenine (Chambon et al., 1966; Nishizuka et al., 1967; Reeder et al., 1967 and Hasegawa et al., 1967). This product of the ADPR-transferase was finally identified as the homopolymer of adenosine diphosphoribose, ADP-ribose units; linked

together through (1"-2') glycosidic bonds between adjacent ribose molecules (Chambon et al., 1966 and Doly & Petek, 1966).

Since the discovery of poly(ADP-ribose), there has been considerable speculation as to its physiological function. The observation that the ADPR-transferase is responsible for modification of chromatin associated proteins led to speculation of its involvement in the regulation of nuclear metabolism. Data accumulated to date have resulted in a correlation between the activity of ADPR-transferase and DNA excision repair (Durkacz et al., 1980). It has also been implicated in a number of examples of eukaryotic cellular differentiation (Farzaneh et al., 1980, 1982; and Williams, 1983a,b, 1984), together with mitogen activation of quiescent lymphocytes (Johnstone & Williams, 1982; Greer & Kaplan, 1983; Johnstone, 1984 and Johnstone & Darling 1985), Sister chromatid exchange (Natarajan et al., 1982), antigenic variation in T.brucei (Cornelissen et al., 1985), and the stable expression of transfected DNA in mammalian host cells (Farzaneh personal communication) have all been related to ADP-ribosylation. For a recent review of ADPR-transferase involvement in cellular processes see Shall (1984); Ueda & Hayaishi (1985); Althaus & Shall (1985); Gaal & Pearson (1985 and 1986). A brief report of circumstantial evidence suggesting that ADP-ribosylation, by implication poly(ADP-ribosylation), may have a role in plant cell differentiation was given by Sugiyama and Komamine (1987).

Studies of the physiological role of ADP-ribosylation have basically relied on the measurements of enzyme activity, quantitation of the in vitro levels of poly(ADP-ribose), and studies of the effect of inhibition of ADPR-transferase activity during various cellular

functions. Alternatively the identification and study, usually in vitro, of ADP-ribosylated proteins has also been looked at. The earliest approach to determine the biological function of poly(ADP-ribose) was to isolate nuclei from cells in various states and attempt to correlate the activity of the enzyme with a particular cell state. One major problem in using isolated nuclei is the fact that the isolation procedure itself leads to formation of experimentally induced DNA strand-breaks which themselves activate the enzyme (Halldorsson et al., 1978). Therefore the validity of such enzyme activity measurements must be treated with caution because it is not a true reflection of the situation in the intact cell. In recent years various systems of cell permeabilisation for both prokaryotic (Scholler et al., 1972; Pisetsky et al., 1972) and eukaryotic cells (Reinhard et al., 1977; Berger et al., 1977; Castellot et al., 1978; Miller et al., 1978) have provided a more realistic model for the study of enzyme activity than the use of isolated nuclei.

In addition to modifying a variety of proteins the ADPR-transferase catalyses an automodification reaction (Kawaichi et al., 1981). In this reaction which is probably intermolecular, the transferase can attach to itself as many as 15 ADP-ribose chains of up to 80 residues each (Kawaichi et al., 1981).

1.3.2 Non-nuclear(ADP-ribosylation) and its Biological Significance

The extranuclear ADP-ribosylation system does not require the presence of DNA or strand-breaks for activity. This ADP-ribosylation is a major cytoplasmic post-translational protein modification. It includes membrane bound (Richter et al., 1983), cytosolic (Iglewski et al., 1984) and mitochondrial protein ADP-ribosylation (Richter et

al., 1983). Richter et al. (1983) postulated a role for ADP-ribosylation of specific inner mitochondrial membrane proteins in the regulation of Ca^{2+} efflux. During their studies on the mechanism of hydroperoxide-induced release of Ca^{2+} from rat liver mitochondria, Richter's group observed the cleavage of intramitochondrial pyridine nucleotides concomitant with the ADP-ribosylation of a 31kD protein at an arginine residue at the inner side of the inner mitochondrial membrane.

Recently the presence of a cytosolic ADPR-transferase has been demonstrated which covalently modifies, and hence inactivates, protein elongation factor 2 (EF2) at a post-translationally modified histidine residue in polyoma virus-transformed BHK cells and bovine liver (Iglewski et al., 1984). Furthermore, Carter has suggested that cAMP production and ADPR-transferase activity may be related in the hormonally responsive MCF-7 human breast cancer cell lines (Carter, 1984). More recently there has been the exciting demonstration, albeit in vitro, of ADP-ribosylation of the human c-Ha-ras protein by isolated hen liver ADPR-transferase (Kawamitsu et al., 1986). Although this modification had no detectable effect on either the GTP binding or GTPase activity of the ras protein it does strengthen the possibility that ADP-ribosylation may play an important role in signal transduction mechanism. Observations such as these indicate that cytosolic, monomeric ADP-ribosylation may play a regulatory role in several cellular processes including transmembrane signal transduction.

The following sections will examine the evidence for the involvement of ADPR-transferase in DNA repair and cellular growth and differentiation in more detail.

1.4 DNA Excision Repair

The most substantial and convincing evidence of a biological role of ADP-ribosylation is that of its involvement in DNA excision repair. It is now known that DNA damaging agents such as UV, γ or X-radiation, dimethyl sulphate (DMS) and methylnitrosourea (MNU) damage DNA which results in activation of ADPR-transferase and depletion of the cellular NAD levels (Shall, 1984). The cellular NAD content decreases most rapidly at the time when ADPR-transferase activity is highest.

Using streptozotocin, which is a glucose derivative of methylnitrosourea, Whish et al. (1975) demonstrated that in isolated nuclei from Physarum polycephalum, ADPR-transferase was activated 3-fold and that the drop in cellular NAD level, induced by DNA-damaging agents is due to activation of ADPR-transferase and is not caused by inhibition of NAD biosynthesis nor by activation of NAD glycohydrolase. Other treatments known to damage DNA have also been shown to stimulate ADPR-transferase activity eg. Davies et al. (1976, 1977) have demonstrated increased enzyme activity in cells treated with γ -radiation and with the polypeptide antitumour antibiotic, neocarzinostatin. As mentioned earlier it is also well documented that ADPR-transferase is totally dependent on the presence of DNA which contains strand-breaks (Tsopanakis et al., 1978; Jankidevi et al., 1974; Miller, 1975b; Halldorsson et al., 1978; Berger et al., 1978b, 1979 and Benjamin & Gill 1980a).

Inhibitors of ADPR-transferase activity inhibit the drop in NAD following DNA damage (Davies et al., 1977, 1978; Jacobson & Jacobson, 1978; Skidmore et al., 1979; Durkacz et al., 1980a,b; Jacobson et al., 1980 and Sims et al., 1982). Inhibitors of ADPR-transferase

also retard the excision repair of DNA damage (Durkacz et al., 1980; Gray et al., 1981; Durkacz et al., 1981a,b; James & Lehman, 1982 & Shall et al., 1982). These inhibitors also perturb the activation of DNA ligase which occurs following DNA damage (Creissen and Shall, 1982). It has also been shown that inhibitors of ADPR-transferase activity enhance the cytotoxicity of DNA damaging agents (Nduka et al., 1980; Durkacz et al., 1980; Durrant et al., 1981 and James & Lehman, 1982).

These observations provided a rational explanation for the drop in cellular NAD and at the same time strongly suggest the involvement of ADPR-transferase in the cytotoxic effects of DNA damaging agents.

1.5 Cellular Growth and Differentiation

The evidence for the possible involvement of ADP-ribosylation in the regulation of DNA synthesis and cell proliferation is both indirect and contradictory. Experiments with permeabilized cell systems have shown that the arrest of DNA synthesis in mouse lymphoma L1210 cells by nutrient starvation, virus infection and by density inhibition of growth results in an increase of ADPR-transferase activity. The transfer of temperature sensitive mutants of chinese hamster ovary (CHO) cells to restrictive temperature, or the treatment of L1210 cells with cytosine arabinoside also results in the increase of the intrinsic activity of ADPR-transferase (Berger et al; 1978 a,b,c). Using permeabilized Xeroderma pigmentosum cells, Berger showed that UV-radiation decreased DNA synthesis but had no effect on poly(ADP-ribose) synthesis in these cells. Upon addition of exogenous UV endonuclease, poly(ADP-ribose) levels increased and DNA synthesis increased to slightly higher than normal. This

increase in DNA synthesis was inhibited by ADPR-transferase inhibitors (Berger and Sikarski, 1981). However, as pointed out by Hilz et al. (1982), it is important to note that the in vivo measurements of mono(ADP-ribose) in hepatoma cells in fact suggest a role for ADP-ribosylation in differentiation rather than cell proliferation. It is also important to note that although there are changes in both the endogenous levels of ADP-ribose and intrinsic activity of ADPR-transferase in different proliferative states and different stages of the cell cycle, the data available do not suggest that ADP-ribosylation is involved in the regulation of DNA synthesis or cell proliferation. In fact a number of different studies have shown that inhibition of ADPR-transferase activity does not affect the rate of DNA synthesis or cell proliferation in mouse lymphoma L1210 cells (Berger et al., 1978a and Durkacz et al., 1980), mouse fibroblasts 3T3 cells (Shall et al., 1982), Chinese hamster ovary cells (Berger et al., 1982) and Trypanosoma cruzi (Williams, 1983b). In the past few years, several types of experiments have implicated ADPR-transferase in cellular differentiation, although earlier studies have been unsuccessful in providing clear evidence of this.

Caplan and Rosenberg (1975) presented pieces of evidence to support the suggestion that the differentiation of mesodermal cells of chick embryo limb buds involved ADPR-transferase. Their use of 3-acetylpyridine has been criticized because this compound was subsequently found to be cytotoxic to peripheral nerves in this system (McLachlan et al., 1976).

Changes in ADPR-transferase activity measured in cell lysates or isolated nuclei have been correlated with differentiation in cardiac muscle (Claycomb, 1976), Friend erythroleukemic cells (Rastl &

Swetty, 1978 and Morioka et al., 1979) and Xenopus laevis embryos (Farzaneh & Pearson, 1979). As mentioned earlier, measurement of ADPR-transferase activity in permeabilized cells is likely to reflect the situation in vivo more closely because the DNA will be less damaged by the experimental system than in isolated nuclei. Changes in ADPR-transferase activity measured in permeabilized cells have been correlated with differentiation in chick muscle development in vitro (Farzaneh et al., 1980,1982), in 3T3-LI pre-adipocytes (Pekala et al., 1981), in HL-60 promyelocytic leukaemic cells (Kanai et al., 1982), and in the induction of foetal enzymes in cultured hepatocytes (Althaus et al., 1982).

A complementary approach has been the quantitation of the reaction products, poly and mono(ADP-ribose) covalently attached to proteins. Bredehorst et al. (1981) reported that foetal liver contained considerably less mono(ADP-ribose) attached to protein than did adult liver. Even earlier in development in X.laevis embryos, mono(ADP-ribose) protein conjugates were present in quantities so small that they were not detectable (Williams et al., 1983a). A similar positive correlation was also found with the degree of differentiation of hepatomas (Hilz, 1981). The quantity of a sub-fraction of mono(ADR-ribose) protein conjugates in which the attachment to protein was not affected by hydroxylamine was found to be related to the differentiation of Dictyostelium discoidum (Bredehorst et al., 1980). The distribution of ADPR-transferase in human blood cells was examined by Ikai et al. (1980a,b). He showed that lymphocyte and monocyte nuclei were capable of synthesising poly (ADP-ribose) but not nuclei from granulocytes or erythrocytes. These results assumed that the synthesised poly(ADP-ribose) served as a marker for differentiation of granulocytes. Young and Sweeny (1978)

showed a decreased incorporation of adenosine in mouse ova during and after fertilization. In 1979 these authors also showed that poly(ADP-ribose) in embryos had a different chain length and linkage than that in unfertilised ova. The quantity of polymer undergoes very large variation during the differentiation of HL-60 promyelocytic leukaemia cells (Kanai et al., 1982), and has been reported to be low in X.laevis early embryos (Williams et al., 1983a). Williams (1983b) has also shown that ADPR-transferase activity is required for the differentiation of Trypanosoma cruzi amastigotes to epimastigotes and trypomastigotes.

The most compelling evidence for the involvement of ADPR-transferase in eukaryotic cell differentiation has been obtained using chemical inhibitors of the enzyme. The interpretation of such experiments clearly depends on the specificity of the inhibitors. Several different classes of ADPR-transferase inhibitors - nicotinamides (Clark et al., 1971), methylxanthines (Levi et al., 1978), and benzamide derivatives (Purnell & Whish, 1980), have been used to strengthen the view that the effects observed in vivo were due to inhibition of ADPR-transferase. Benzamide derivatives display the greatest physiological specificity (Purnell & Whish, 1980) and have been used most widely. Two important controls for inhibitor specificity in these experiments are comparisons of their effects on differentiation with that on a simple cycle of proliferation, and the use of non-inhibitory chemical analogues. Farzaneh et al. (1980, 1982) first used such inhibitors of ADPR-transferase in studies of chick myoblast differentiation in vitro. The mitogen-induced differentiation of human peripheral-blood T-lymphocytes is also blocked by inhibitors of ADPR-transferase (Johnstone & Williams, 1982). Investigation of the morphological differentiation of the

protozoan parasite Trypanosoma cruzi has indicated that benzamide derivatives, nicotinamides and methylxanthines all blocked the extracellular and intracellular differentiation of Trypanosoma cruzi (Williams, 1983b). Further recent studies have shown that the rate of antigenic switching in 3-aminobezamide treated rats is reduced 15-fold over controls (Cornelissen et al., 1985). Antigenic switching is a process which involves DNA strand-breaks. ADPR-transferase is required for the repair of the DNA breaks induced by this process and reduction of antigenic switching rate by 15-fold, indicates that this aspect of ADPR-transferase activity is also blocked by 3-aminobenzamide.

These results strongly indicate the involvement of ADP-ribosylation in differentiation.

1.6 Poly(ADP-ribose) Degradation

There are two classes of enzymes which catalyse the degradation of poly(ADP-ribose): they are poly(ADP-ribose) glycohydrolases and poly(ADP-ribose) phosphodiesterases.

Poly(ADP-ribose) glycohydrolase, degrades the polymer to release monomeric ADP-ribose. It has been found in all the tissues studied (Purnell et al., 1980), and is the only enzyme which has been reported to specifically act on poly(ADP-ribose). This glycohydrolase cleaves the glycosidic ribose-ribose bonds leaving the terminal ADP-ribose attached to protein (Ueda et al., 1972 and Miwa et al., 1974) (Fig. 1.4). Poly(ADP-ribose) glycohydrolase does not act on mono(ADP-ribose) which contains no glycosidic bonds. In rat liver nuclei the last ADP-ribose molecule is removed by an enzyme

called ADP-ribose histone hydrolase (Okayama et al., 1978) (Fig 1.4). This enzyme activity is heavily inhibited by denatured DNA, but to a much lesser extent by double-stranded DNA (Stone et al., 1978). Tavassoli et al. (1983) purified a poly(ADP-ribose) glycohydrolase from pig thymus. Recently Tanuma et al. (1986a,b,c) reported the purification of one form of poly(ADP-ribose) glycohydrolase from guinea pig liver nuclei and human erythrocytes and, interestingly, two apparently distinct poly(ADP-ribose) glycohydrolase activities from HeLa cells.

In contrast to poly(ADP-ribose) glycohydrolases, (ADP-ribose) phosphodiesterases have only been found in rat liver (Futai et al., 1967), cultured cells of Nicotina tobacum (Miwa et al., 1975) and snake-venom (Chambon et al., 1966; Nishizuka et al., 1967; Fujimura et al., 1967). Snake-venom phosphodiesterase was the first enzyme known to hydrolyse the pyrophosphate bond between the two ribose moieties of ADP-ribose (Fig. 1.4). In this reaction one molecule of 5'-AMP is produced from the 5' end of the polymer and n molecules of phosphoribosyl-AMP (PR-AMP) from the internal residues. One ribose-phosphate molecule is left covalently attached to the protein. Hayaishi et al. (1985) review the removal of the initial ADPR residue.

Poly(ADP-ribose) can be unambiguously identified by its digestion products, 5'-AMP and PR-AMP. These can be separated by paper chromatography (Nishizuka et al., 1969) or thin layer chromatography (Stone et al., 1973). No degradation of poly(ADP-ribose) takes place by deoxyribonuclease (DNase), ribonuclease (RNase), spleen phosphodiesterase or micrococcal nuclease (Hasegawa et al., 1967).

Pa=Protein acceptor

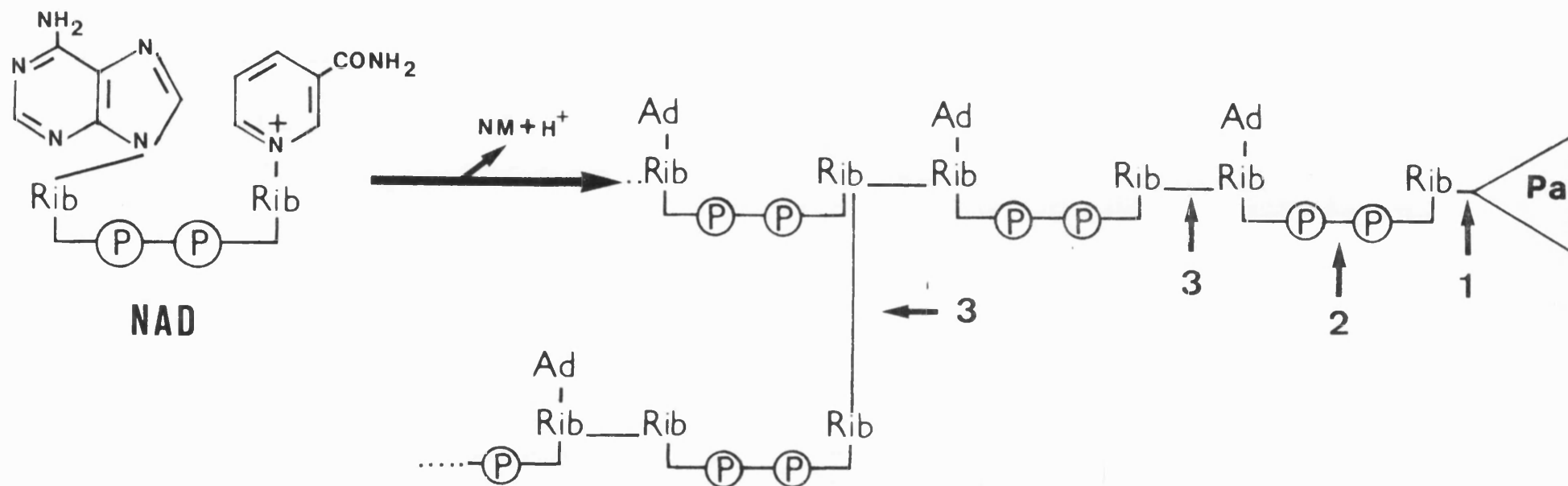


Fig. 1.4 Degradation of poly(ADP-ribosyl) protein

Fig. 1.4 Enzymes Involved in the Degradation of Poly(ADP-ribose)

1. ADP-ribosyl hydrolase (eg. histone hydrolase)
2. Posphodiesterase
3. ADPR-glycohydrolase

1.7 ADPR-transferase Inhibitors

Several groups of compounds competitively inhibit the activity of nuclear ADPR-transferase. These include nicotinamides, NAD analogues, thymidine and some of its analogues, methylxanthines particularly theophylline (Levi et al., 1978) and benzamides. Nicotinamide was one of the first inhibitors of nuclear ADPR-transferase discovered. Nicotinamide inhibits not only the nuclear ADPR-transferase activity but also the cytoplasmic NAD glycohydrolase activity and several other enzymes not related to the NAD metabolism these include cAMP phosphodiesterase (Shimoyama et al., 1972) and tRNA methylase (Buch et al., 1972). More importantly, nicotinamide is a precursor for NAD. Administration of nicotinamide to intact cells may therefore both competitively inhibit the ADPR-transferase activity and also stimulate its activity by increasing the substrate concentration. Therefore nicotinamide is not a suitable compound for the study of ADPR-transferase inhibition in vitro. However since nicotinamide is a precursor for NAD, nicotinamide starvation of intact cells can be used as a means to reduce the cellular NAD level and thus inhibit ADPR-transferase activity by substrate depletion (Jacobson et al., 1979; Jacobson & Narasimhan, 1979 and Durkacz et al., 1980).

Nicotinamide analogues are also inhibitors of nuclear ADPR-transferase activity. Studies with these compounds have suggested that the essential structure for ADPR-transferase inhibition is a planar, uncharged ring with a carboxylic group (Purnell & Whish, 1980). 5-methyl-nicotinamide is a nicotinamide analogue commonly used in ADPR-transferase studies. This compound is not incorporated into NAD, and is not known to have any physiological effect apart from ADPR-transferase inhibition (Clark et al., 1971 and Kidwell &

Burdett, 1974).

The substrate for ADPR-transferase is β -NAD. However, at high concentrations β -NAD inhibits the enzyme, suggesting negative cooperativity (Poirier et al., 1978). The enzyme is also inhibited by substrate analogues such as α -NAD and NADH₂ (Preiss et al., 1971). 2'-deoxy NAD and 3'-deoxy NAD can be used as substrate in foetal rat liver nuclei and Hela cells nuclei giving rise to the synthesis of poly(ADP-ribose) molecules which are both structurally and functionally altered (Suhadolnik et al., 1977, 1980).

Thymidine and a number of its analogues, including thymine, bromodeoxyuridine, bromo-uridine (Preiss et al., 1971), and ribothymidine (Shall, 1975) inhibit nuclear ADPR-transferase activity, though the degree of inhibition varies widely between the inhibitors.

The methylxanthines, caffeine, theobromine and theophylline also inhibit nuclear ADPR-transferase activity (Claycomb, 1976; Davies et al., 1978 and Levi et al., 1978). In this inhibition theophylline and theobromine are an order of magnitude better than caffeine (Shall et al., 1977). Theobromine and thymidine have been reported to inhibit NAD-glycohydrolase of rabbit reticulocytes (Wu et al., 1978) but not of mouse lymphoma L1210 cells (Skidmore et al., 1979).

The best inhibitors of nuclear ADPR-transferase activity so far discovered are benzamides especially 3-aminobenzamide, 3-methoxybenzamide (Purnell & Whish, 1980) and 3-acetamidobenzamide (Purnell & Whish, 1980). All three of these compounds are powerful inhibitors of ADPR-transferase activity. Furthermore they do not significantly inhibit NAD-glycohydrolase or poly(ADP-ribose)

glycohydrolase activity (Purnell & Whish, 1980). These inhibitors also do not inhibit cell growth or the synthesis of RNA, DNA or proteins (Purnell & Whish, 1980; Durkacz et al., 1980 and Williams 1983a,b). Furthermore their acid analogues, 3-aminobenzoic acid and 3-methoxybenzoic acid do not inhibit ADPR-transferase activity at concentrations several times higher than the corresponding amide (Purnell & Whish, 1980 and Durkarz et al., 1980).

The effectiveness of the inhibitors varies. These are listed in table (1.2) along with their K_i (inhibition-constant) values, and the cells or tissues used in the experiments used to derive the K_i values. See Figs. (1.5 & 1.6) for the structure of ADPR-transferase inhibitors.

It is important to note that despite wide spread use of these inhibitors in ADPR-transferase studies Cleaver and co-workers have demonstrated the interference of the benzamide analogue inhibitors, and some of their non-inhibitory analogues, with a number of other biochemical processes, particularly nucleotide biosynthesis (Cleaver et al., 1983, Milam & Cleaver, 1984). Furthermore, Cleaver and Morgan (1987) in a recent report have proposed that physiologically acceptable concentrations of the inhibitors actually accelerate DNA excision repair.

However, none of the non-specific effects described by Cleaver et al., are common to all the available inhibitors. At the same time, none of the non-specific effects are restricted only to the inhibitory analogues; they are often also observed in response to treatment with the non-inhibitory analogues. Therefore if a number of inhibitors, together with their non-inhibitory analogues, are used at non-toxic concentrations, and all the inhibitors but none of the

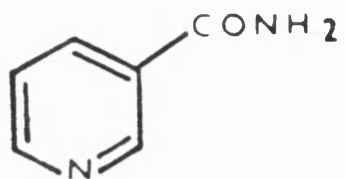
non-inhibitors show the same biological effect, this is likely to be the result of ADPR-transferase inhibition rather than the product of disparate effect of each of the inhibitors on other biosynthetic processes.

Table 1.2

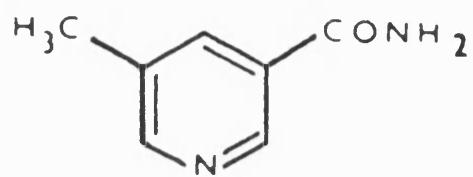
<u>INHIBITOR</u>	<u>ki (μM)</u>	<u>TISSUE OR CELLS</u>	<u>REFERENCES</u>
Nicotinamide	52	Ehrlich ascites tumor cells	Romer et al. (1968)
	20	Rat liver nuclei	Clark et al. (1971)
	5.5	Physarum polycephalum	Brightwell (1973)
	14.3	Ln cell nuclei	Stone&Shall (1973)
	13.0	L5178Y cells (mouse lymphoma)	Halldorsson et al. (1978)
Picolinamide	-	N. human lymphocytes	Sims et al. (1982) Yamamoto & Kamoto (1980)
5-Methyl-nicotiamide	200	Rat liver nuclei	Clark et al. (1971)
	30.0	L5178Y	Halldorsson et al. (1978)
Thymidine	32.5	Ln cells	Ueda et al (1972) Stone et al (1973)
5-Bromo-2'-deoxyruacil	-	-	Khan (1977)
Methyxanthines	-	Rat cardiac muscle	Claycomb (1976)

Table 1.2 Continued:

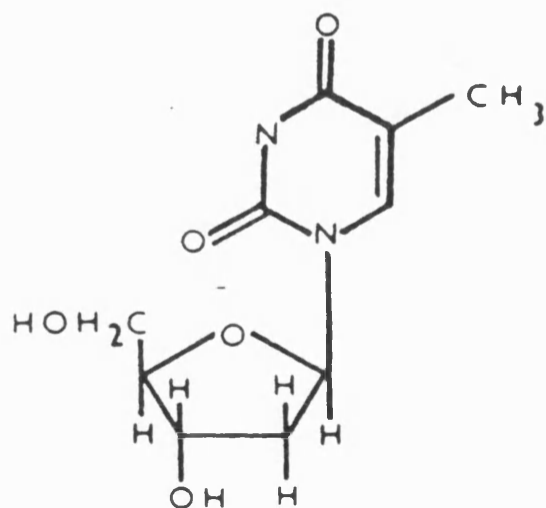
<u>INHIBITOR</u>	<u>ki (μM)</u>	<u>TISSUE OR CELLS</u>	<u>REFERENCES</u>
Theophylline	15.2	L5178Y cells	Halldorsson et al. (1978) Levi et al. (1978)
Theobromine	29.8	-	Halldorsson et al. (1978)
Caffeine	244	-	"
Benzamide	-	-	Shall (1975)
Pyrazinamide	-	-	Khan (1977)
3-Aminobenzamide	2.6	Pig thymus	Purnell et al. (1980)
3-Aminobenzmide	4.3	In permeabilised <u>T.brucei</u>	Farzaneh et al. (1985)
3-Aminobenzoic acid	-	-	"
3-acetamidobenzamide	0.43		Prunel & Whish (1980)



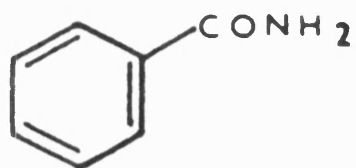
NICOTINAMIDE



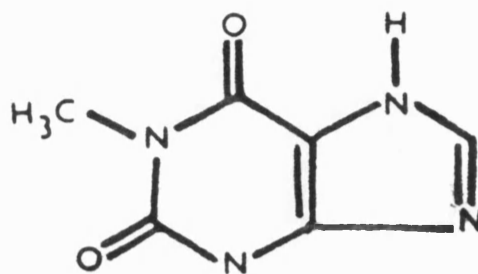
5-METHYL-NICOTINAMIDE



THYMIDINE

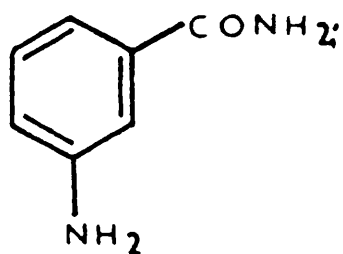


BENZAMIDE

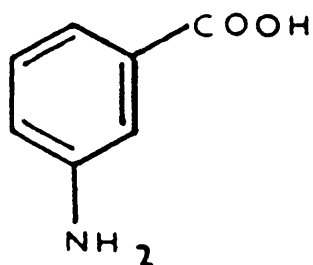


THEOPHYLLINE

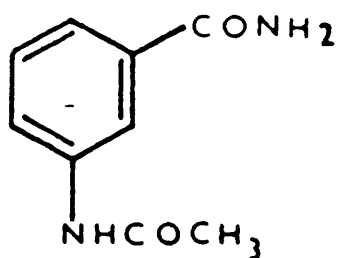
Fig. 1.5 Inhibitors of ADPR-transferase



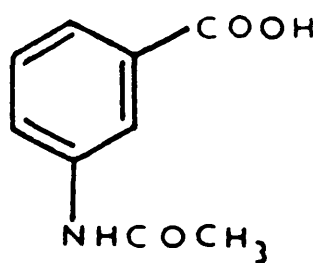
3-AMINOBENZAMIDE



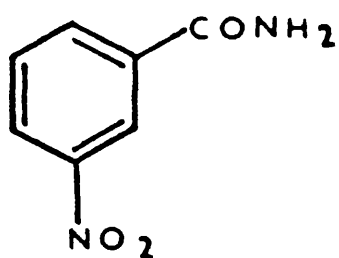
3-AMINOBENZOIC ACID



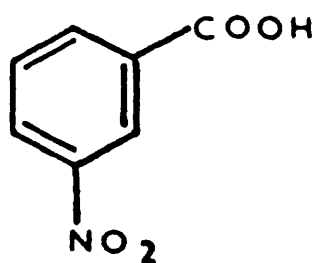
3-ACETAMIDOBENZAMIDE



3-ACETAMIDOBENZOIC ACID



3-NITROBENZAMIDE



3-NITROBENZOIC ACID

**Fig. 1.6 Inhibitors of ADPR-transferase
and their acid analogues**

CHAPTER TWO

Introduction to Trypanosomes

2. CLASSIFICATION OF TRYPANOSOMES

Kingdom	Animalia
Sub-kingdom	Protozoa
Phylum	Sarcomastigophora
Class	Zoomastigophora
Order	Kinetoplastida
Family	Trypanosomatidae
Genus	Trypanosoma
Group	<div><div>Stercoraria</div><div>Salivaria</div></div>
Subgenus	<div><div>Schizotrypanum</div><div>Trypanozoon</div></div>
Species	<div><div><u>T.cruzi</u></div><div><u>T.brucei</u></div></div>
Subspecies	<div><div></div><div><u>T.brucei.gambiense</u> <u>T.brucei.rhodesiense</u> <u>T.brucei.brucei</u></div></div>

(Various authors).

2.1 General Background

Trypanosomes are simple unicellular eukaryotes and among the most successful parasites of man and his domestic animals. Mankind is subject to infection with three different trypanosomes, Trypanosoma brucei, Trypanosoma cruzi and Trypanosoma rangeli. All three are insect vector borne but the behaviour of these parasites in their hosts, human and insect, is strikingly dissimilar.

Trypanosoma brucei multiplies in the blood and other fluids of a variety of mammals, game and cattle as well as man and is one of the numerous species transmitted by tsetse flies in Africa. Tsetse flies are distributed throughout East, Central and West Africa over an area embracing 38 countries and covering approximately 10 million Km². Only certain stocks of the parasite are able to infect man; these are at all times morphologically indistinguishable from their nonhuman-infective counterparts which are assigned to the subspecies Trypanosoma brucei brucei (T.b.brucei). T.b.brucei is nonhuman-infective because in human serum there is a factor responsible for selective lysis of this parasite. This factor has been identified as high-density lipoprotein (Rifkin, 1978). The ability of human serum to induce lysis of T.b.brucei, but not T.b.rhodesiense and T.b.gambiense forms the basis of the in vitro and in vivo infectivity tests (Rickmann and Robson, 1970 and Hawking, 1973). Man-infecting T.brucei causes sleeping sickness. Stocks which cause the acute East African form of the disease are designated T.brucei rhodesiense and those that induce the chronic West African version, T.brucei gambiense. Broadly speaking the ecology of the tsetse vectors of these two subspecies is different (Jordan, 1985). For all three subspecies, (T.b.brucei, T.b.rhodesiense and T.b.gambiense), however, transmission is cyclical; the parasites ingested by the blood-sucking

insect (tsetse fly) undergo bouts of multiplication, change in form; with migration from gut to salivary glands where they become mammal-infecting metacyclic trypanosomes to be inoculated with the fly's saliva during feeding.

The causative agent of Chagas' disease Trypanosoma cruzi, multiplies inside host cells which may be mononuclear phagocytes and muscle. This gives rise to motile trypanosomes which emerge from their intracellular habitat to find their way to other cells. They may be picked up by the vector, a blood-sucking insect of the family Reduviidae. Parasite multiplication and development to the metacyclic stage take place wholly within the gut of the vector, the metacyclic stage being voided with the faeces when the insect takes a blood meal and gaining entry by contamination of the wound. In their mode of transmission, T.brucei and T.cruzi represent the two major subdivision of mammalian trypanosomes, the Salivaria and Stercoraria (Hoare, 1972); the former is characterized by transmission via the anterior station (i.e. inoculation) and the later by posterior station (contaminative) transmission.

The third species, T.rangeli does not reproduce in its human host and is therefore non-pathogenic. It is of medical importance only in that it confounds diagnosis of Chagas' disease by being present in the same vector species and having the same distribution.

2.2 The Life Cycle of T.brucei

T.brucei is transmitted by the tsetse fly Glossina, within which it undergoes important developmental changes (Robertson 1912; Vickerman 1965). Electron microscope studies of the successive stages in the life cycle of T.brucei reveal that underlying the

obvious morphological alterations more complex changes in the parasite's architecture are taking place (Vickerman, 1985) (Fig. 2.1). The most striking changes occur in the mitochondrial system and in the surface membrane of the flagellum (Vickerman 1971). Both can be related to the survival mechanisms of the parasite, the former to adaptive changes in its energy metabolism, the latter to its invasion of the mammalian host's non-specific defence mechanisms. Other changes occur in the endocytotic apparatus, which take up protein and fluid (Steiger, 1973), and the glycosomes. The latter are organelles seemingly peculiar to the kinetoplast-bearing flagellates being the site of glycolysis in these organisms (Oppenheimer, 1977).

The tsetse fly ingests parasites when taking a blood meal from an infected animal. In the insect midgut the trypanosomes then differentiate into procyclic forms. The procyclics lose the variable surface glycoprotein (VSG) that form the surface coat in the animal bloodstream forms (Barry, 1979). They are noninfectious to the animal host, and undergo drastic changes in metabolism. After about three weeks, these parasites migrate to the insect salivary gland where they develop ultimately into metacyclic forms. These metacyclics morphologically resemble the animal bloodstream form. They are infectious to animals and they express the variant surface glycoprotein (Vickerman, 1969; LeRay 1978). When the fly bites another animal the parasites present in its saliva are injected into the blood where they quickly develop into long slender bloodstream forms. These cells divide rapidly by binary fission, alter their metabolic pathways, and continue to express variant surface glycoproteins. They begin to undergo antigenic variation. As the infection proceeds, the long slender T.brucei, differentiates further

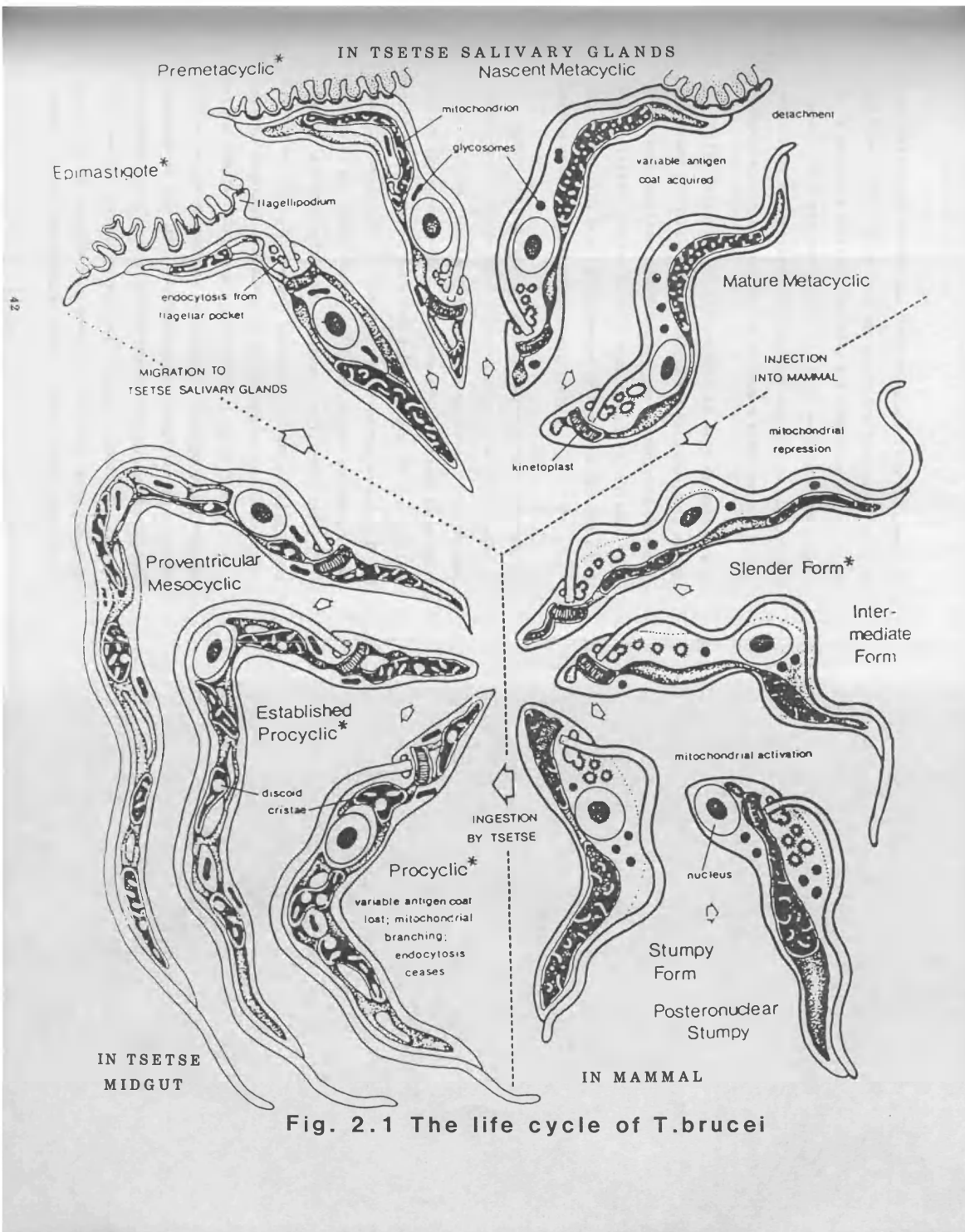


Fig. 2.1 The life cycle of *T. brucei*

into short-stumpy bloodstream forms (Robertson, 1912; Vickerman, 1965 and Wijers 1957). These forms are nondividing (Robertson, 1912), and it is generally believed that they are preadapted to life in the insect. After the tsetse takes its blood meal from the infected animal, the short-stumpy trypanosomes complete the life cycle by differentiating into procyclics in the insect midgut (Wijers, 1960).

Differentiation of pleomorphic as well as monomorphic populations of rodent-adapted strains which have a uniform slender morphology (Bohringer & Hecker, 1974) can be studied in various in vitro systems (Tobie, Brand & Mehlman, 1950; Pitman, 1970; Cunningham, 1977; Brun & Schonenberger 1979; Barry & Vickerman, 1979; Ghiotto et al., 1979 and Bienen et al., 1981).

Some of the complex morphological, ultrastructural and metabolic changes which characterize differentiation have been studied in detail. First, the repression of the synthesis of the variant surface glycoprotein (VSG) in the coated bloodstream forms is an early event which is followed by coat release to form coatless procyclic cells (Barry & Vickerman, 1979; Ghiotto et al., 1979 and Overath et al., 1983). Second, differentiation involves profound changes in energy metabolism (Bowmann & Flynn 1976; Hill, 1976; Oppendoes, 1985). In mammal, bloodstream forms of T.brucei are entirely dependent on glucose for energy supply. The human bloodstream contains glucose at approximately 1 mg/ml (5 mM) and this constant source of substrate eliminates the need for energy reserves (Oppendoes et al., 1976). In long slender bloodstream forms glucose is degraded solely by glycolysis to form two mol pyruvate/mol glucose under aerobic conditions (Brohn & Clarkson, 1978). The NADH in glycolysis is reoxidised by means of mitochondrial glycerol-3-phosphate oxidase and molecular oxygen. The pro-mitochondrion lacks

a functional citric acid cycle as well as a cytochrome-linked respiratory chain. Stumpy forms contain some mitochondrial enzymes, such as 2-oxoglutarate dehydrogenase and proline oxidase (Vickerman, 1965). During differentiation to procyclic cells a functional respiratory chain containing cytochromes is formed (Bienen, Hammadi & Hill, 1981; Bowmann & Flynn, 1976; Hill, 1976; Vickerman, 1965; Ryley, 1962; Srivastava & Bowman, 1971, 1972; Evans & Brown, 1972 and Brown et al., 1973). Synchronous differentiation requires two external signals, a temperature change from 37°C to 27°C and the addition of TCA cycle intermediates cis-aconitate and/or citrate as inducers (Brun & Schonenberger, 1981). Established procyclic cultures oxidize glucose mainly to CO₂ (Ryley, 1962) while proline can also be metabolized at a high rate to glutamate, alanine, CO₂ and several other products (Ford & Bowman, 1973). These metabolic changes are considered to reflect the transition from glucose, as the main energy source of bloodstream forms in the mammal, to amino acid or peptide based diet for the procyclics in the midgut of the fly. The observation of Brun and Schonenberger (1981) that citrate and/or cis-aconitate stimulated differentiation led to the suggestion that these metabolites serve as activators or inducers of mitochondrial enzymes.

2.3 Kinetoplast DNA

The first indication of the existence of kinetoplast DNA came about 75 years ago with the discovery of a structure near the base of the trypanosome's flagellum that stained brightly with basic dyes. This structure, at one time presumed to be involved in cell motility, was named the kinetoplast.

The kinetoplast of trypanosomes is a mass of mitochondrial DNA (K-DNA), peculiar in that it is composed of a single network of interlocked circular DNA molecules of two size classes (Englund, 1981). The maxicircles (contour length 22 μm in T.brucei, 33 μm in T.cruzi) are present as 25-50 copies per network, and the minicircles (contour length 1.0 μm in T.brucei, 1.4 μm in T.cruzi) as 5000-10,000 copies. Maxicircle DNA contains genetic information analogous to that of other mitochondrial DNAs as it hybridises with mitochondrial gene sequences from other organisms, specifically, cytochrome oxidase subunits I and II, cytochrome b, and the Oligomycin sensitive ATPase. Maxicircles also hybridise trypanosome mitochondrial ribosomal RNA. The minicircles are heterogeneous in sequence, evolve exceedingly rapidly in a given stock, and do not appear to be transcribed. Despite a great deal of attention in recent years, their function other than holding the maxicircles together in a network, is unknown (Stuart, 1983). During cell division the K-DNA network replicates after the flagellar basal body and before nuclear division (Hajduk, et al., 1984). It is presumed that the reason for association of the amplified mitochondrial genome with the morphogenetic centre of the flagellar base is to ensure segregation of the maxicircles at division, the maxicircle gene products being necessary for activation of the mitochondrion. Mutants of T.brucei which can not differentiate to the procyclic stage (i.e. undergo development in the vector) may show maxicircle deletions or complete absence of maxicircles. Some of the so-called dyskinetoplastic trypanosome lines, arise by failure of network segregation and lack a stainable kinetoplast; neither maxicircles nor minicircles can be isolated from them. No trypanosome lines that have maxicircles but no minicircle network are known (Englund, et al., 1982).

2.4 Antigenic Variation in African Trypanosomes:

Antigenic variation is a powerful survival strategy adopted by certain species of parasitic protozoa to allow them to survive in the immunized host. It is exemplified by the African trypanosomes, which provide the best characterized and most studied system of this kind.

During infection of a mammalian host, African trypanosomes are covered by a surface protein coat. In electron micrographs this surface coat appears as a 12-15nm thick layer (Vickerman, 1969; Vickerman and Luckins, 1969). It was shown, by chemical analysis, to be composed of one species of protein, the variant surface glycoprotein (VSG) (Cross, 1975). Antigenic variation is expressed through the VSG. Early studies revealed that VSGs isolated from different Variant Antigen Types (VATs) differ dramatically in iso-electric point, carbohydrate content, amino acid composition and amino acid sequence of the N-terminal region (Cross, 1975; Johnson & Cross 1977, 1979). African trypanosomes, like T.brucei, survive in the bloodstream of their mammalian host by periodically changing the antigenic identity of their cell surface coat (Vikerman, 1969 & 1978). The coat completely covers the bloodstream form of the organism, but is absent from the parasite during its stay in the midgut of the insect (Vickerman, 1969). In the bloodstream, the surface coat is therefore the only part of the live parasite that is recognized by the host. However, the host's immune defence falls short in completely eliminating the parasites, because a few trypanosomes replace their coat by a different VSG, a process known as antigenic variation (Englund, et al., 1982 and Turner, 1984). This process of antigenic variation has been studied in most detail in Trypanosoma brucei.

Estimates based on gene cloning and DNA hybridization suggest that each trypanosome contains 1000-2000 variant surface glycoprotein genes (Van der ploeg et al., 1982). The frequency of the switching from one antigen type to another has been quoted as between 10^{-4} and 10^{-5} changes/cell generation (Van Meirvenne et al., 1975a and Doyle, 1980). Based on mathematical calculation, Gretel, et al. (1986), reported that most likely switching frequencies range from 1.4×10^{-7} to 3.5×10^{-6} changes/cell generation. This VSG switching is independent of antibody pressure (Doyle et al., 1980) but the mechanisms regulating sequential VSG gene expression are unknown (Borst & Cross, 1982). Only one VSG gene is expressed at any time, although inactivated genes may be re-expressed (Michels, et al., 1984). The importance of antigenic variation to the trypanosome is underscored by the estimate that up to 10% of the trypanosome genome may be devoted to variant antigen genes (Van der Ploeg et al., 1982). Different antigenic types of trypanosomes follow each other during a chronic infection of a mammal in a non-random order (Van Meirvenne, et al., 1975a,b; Capbern, et al., 1977 and Miller & Turner, 1981). The cycle usually continues until death of the host in the absence of any chemotherapy. Cornelissen, et al., 1985, developed an experimental system that allowed the continuous maintenance of a high blood level of 3-aminobenzamide, a competitive inhibitor of the enzyme ADPR-transferase, in rats infected with T.brucei expressing VSG gene 118. The average switching rate found was 2×10^{-6} in controls and 1.3×10^{-7} in 3-aminobenzamide-treated rats (15-fold reduction).

2.5 In vitro Culture and Differentiation of T.brucei:

a) Procyclic forms: The procyclic forms found in the midgut of

tsetse fly vector are non-infective for mammalian hosts and lack a surface protein coat. Today procyclic forms can easily be produced in large quantities in complex (Trager, 1978), semi-defined (Brun & Schonenberger, 1979; Cunningham and Honigberg, 1977 and Steiger & Steiger 1976) or even in a defined medium (Cross & Manning 1973).

Cultures of procyclic forms are usually initiated with bloodstream forms from a mammalian host. The bloodstream forms, mainly the intermediate and stumpy forms (Ghiotto, et al., 1979), normally differentiate within 48 hours to forms morphologically very similar to procyclic forms. The biochemical differentiation, however, has been suggested to proceed more slowly and it takes about four weeks before an established culture is produced (Bienen et al., 1981).

A significant increase in the rate of differentiation can be achieved by the addition of tricarboxylic acid cycle intermediates to the culture medium (Brun & Schonenberger, 1981). Citrate and cis-aconitate in a 3 mM concentration have proved to be the two intermediates which are best able to stimulate differentiation. Another way to obtain a procyclic culture is the direct transfer of midgut forms from an infected Glossina into culture medium.

b) Metacyclic forms: The metacyclic forms are produced in the salivary glands of the vector. It is this stage in the life-cycle of the parasite which is infective to the mammalian host. The in vitro production of metacyclic forms is impeded by the fact that this parasite stage does not divide. Moreover, metacyclic forms differentiate in vitro within 8-10 hours to slender bloodstream forms (Brun, et al., 1984). Metacyclic forms seem to be inhibited from differentiation in the salivary glands by some unknown factor, most

probably a constituent of the salivary secretion.

It has been shown that, when non-infective procyclic forms are cultivated at 28°C in the presence of head-salivary gland explants of Glossina morsitans morsitans infective metacyclic forms are produced (Cunningham & Honigberg, 1977 and Cunningham & Taylor, 1979). Large numbers of explants per culture are required and the percentage of metacyclic forms is normally below 0.1%. It has been demonstrated by antigenic analysis and ultrastructural studies, that these forms are true metacyclics (Gardiner, et al., 1980a,b).

c) Bloodstream forms: The first continuous cultivation of bloodstream forms was achieved by Hirumi et al. (1977a,b) using a bovine fibroblast culture as feeder layer in a tissue-culture medium supplemented with fetal bovine serum. The co-cultivation of trypanosomes and mammalian cells has proved to be essential for the cultivation of the bloodstream forms. Many cell lines isolated from various mammals either of the fibroblast or the epithelial cell type have now been successfully used as feeder cells for the cultivation of T.brucei and T.rhodesiense (Hirumi et al., 1977a,b; Brun, et al., 1979; Hill, et al., 1978a,b; Brun, et al., 1981). The role of the feeder cells is still unclear. Fibroblast conditioned medium does not support growth of the parasite, but allows maintenance of bloodstream forms for up to 72 hours. Separation of fibroblasts and trypanosomes by membranes also results in a loss of growth of the trypanosomes (Tanner, 1980). Primary murine bone marrow cultures also have been shown to support the continuous growth of T.brucei bloodstream forms (Balber, 1983). However Baltz, et al., 1985; have reported a culture system in vitro which supports the rapid growth of infective forms of T.brucei, T.evansi, T.rhodesiense and T.gambiense without using a feeder layer. In this system the presence of

pyruvate and a reducing agent such as 2-mercaptoethanol, was essential.

Bloodstream forms grown in vitro are very similar to the forms found in the mammalian host with regard to ultrastructure (Hecker & Brun, 1982) and infectivity for the mammalian host (Schoni, et al., 1982). They also seem identical in their ability to successively express their different variant surface glycoproteins. This results in antigenic variation in vitro (Doyle, Hirumi and Hirumi, 1978 & 1980).

The relatively low trypanosome densities in all existing bloodstream form cultures (about 10^6 /ml) impedes the mass production of this stage in the life cycle of T.brucei.

2.6 ADPR-transferase Activity in Protozoan Parasites

To date there has been no direct studies on the mechanism of DNA repair in African trypanosomes. However the presence of ADPR-transferase activity in protozoan parasites Plasmedium yoelii (Okolie & Onyezili, 1983), T.cruzi (Williams, 1983b), and T.brucei (Farzaneh et al., 1985; Alsharif et al., 1986a,b) has been reported. Inhibition of ADPR-transferase activity blocks the differentiation, but not the proliferation, of T.cruzi amastigotes to epimastigotes and trypomastigotes (Williams, 1983b). The detection of ADPR-transferase activity in T.brucei, its activation by DNA damage and its inhibition by the inhibitors of this enzyme in higher eukaryotic cells has also been reported (Farzaneh et al., 1985; Alsharif et al., 1986a,b). Studies have also shown that ADPR-transferase activity plays an important role in the process of antigenic switching of T.brucei (Cornelissen et al., 1985). Most switches require the

duplicative transposition of a variable surface glycoprotein (VSG) gene, which involves strand-breaks in DNA and subsequent repair. Cornelissen et al., 1985, showed that the rate of antigenic switching in 3-aminobenzamide treated rats is reduced 15-fold over control. This result indicates that an active ADPR-transferase is required either for the gene switching process or for the repair of the DNA strand-breaks formed during this process.

2.7 Existing Chemotherapy

Several control measurements have been tried for trypanosomiasis, including eradication of tsetse fly habitats, eradication of tsetse flies, eradication of the reservoir hosts of trypanosomes (wild animals), chemoprophylaxis and /or diagnosis followed by chemotherapy.

Chemotherapy of African trypanosomiasis centres on three key drugs: pentamidine for chemoprophylaxis; Suramin for treatment of the early stages of the disease; and melarsoprol for treatment of late stages when trypanosomes are present in the CNS (Williamson, 1962; Hawking, 1963; Williamson, 1970; Apted, 1970; Waddy, 1970; Williamson, 1976; Apted, 1980 and Evans, 1981).

a) Pentamidine: This aromatic diamidine (Fig. 2.2a) was introduced in the late 1940s. Two salts of the drug are in use: pentamidine isothionate (M&B 800) and pentamidine methanesulphonate (Iomidine). The dosages used and effects of the two are similar.

The mechanism of action of aromatic diamidines is still far from resolved. They appear to exert a much more marked effect on biosynthesis, especially of nucleic acids, than they do on catabolism

(Gutteridge, 1969). They are all positively charged at physiological pH and as such bind readily to DNA, though not by an intercalative mechanism (Newton, 1974). Such a binding would explain their effects on nucleic acids generally but not why the synthesis of kinetoplast DNA is particularly sensitive to these drugs (Brack, et al., 1972). Selectivity appears to be due to differential permeability between host and parasite: trypanosomes contain a pentamidine transport system which raises the intracellular concentration of drug to many times the plasma concentration (Damper & patton, 1976).

b) Suramin: This sulphated naphthylamine (Synonyms: sodium suramin, Antrypol, Bayer 205, Belganyl, Fourneau 309, Gemanin, Moranyl, Naganol, Naphuride) (Fig. 2.2b) was brought into general use in the early 1920s and remains unique among anti-trypanosomal drugs in having a net negative charge at physiological pH.

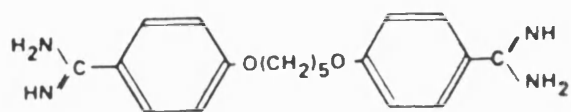
The mode of action of suramin is still uncertain, partly because its slow action makes study in vitro difficult, and partly because it is known to inhibit a multitude of enzymes in cell free systems at millimolar concentrations (Fairlamb & Bowman, 1977 & 1980).

These are two major limitations to suramin. First, it does not pass from the blood into the cerebrospinal fluid and therefore has no curative effect on the disease once the CNS has become involved. Second it suffers from a range of serious side effects.

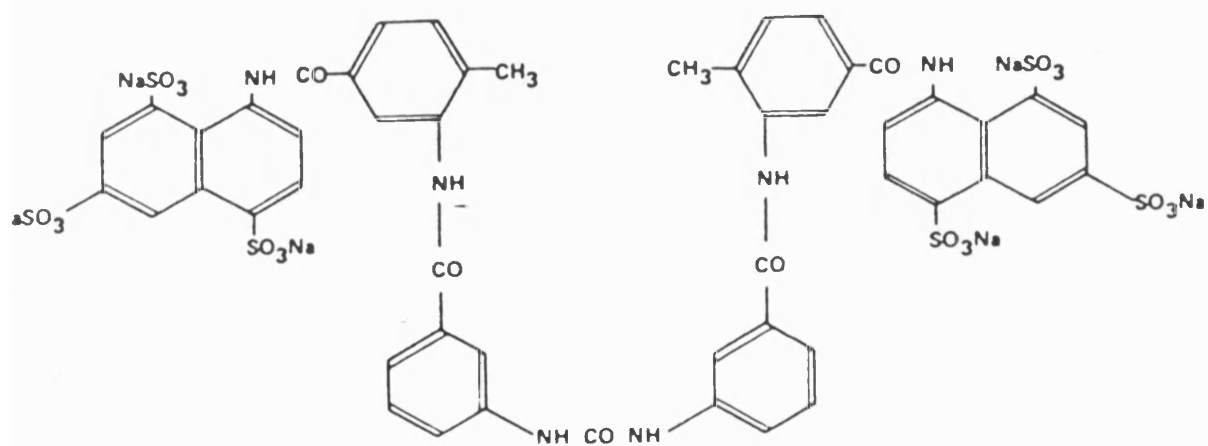
c) Melarsoprol: This arsenical (synonyms: Mel B, Melarsen oxide BAL, Arsobal) (Fig. 2.2c), was introduced in the 1940s.

Melarsoprol, like all anti-trypanosomal arsenicals, is believed to work primarily by disruption of energy generation. Trivalent arsenicals generally have a high affinity for sulphydryl groups,

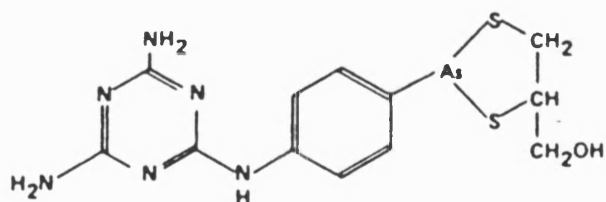
which form the active sites of many enzymes, especially kinases. The study of the anti-trypanosomal arsenicals showed that the glycolytic kinase, hexokinase, phosphokinase and pyruvate kinase, were all particularly sensitive to inhibition (Flynn & Bowman, 1974). There is also another report of a successful clinical trial of the polyamine biosynthesis inhibitor, α -difluoromethylornithine in 17 patients with African trypanosomiasis (Sjoerdsma, Golden and Schechter, 1984).



a-Pentamidine



b - Suramin



c-Melarsoprol

Fig 2.2 Drugs currently used to treat human trypanosomiasis

2.8 AIMS OF THE PROJECT

The aims of this project have been the investigation of the possible involvement of ADP-ribosylation in DNA repair, growth and differentiation of T.b.brucei in vitro. These studies have demonstrated the potentiation of the toxicity of both a monofunctional alkylating agent, dimethyl sulphate (DMS), and ionising radiation (γ -radiation) in T.b.brucei, thus suggesting the involvement of this enzyme in DNA repair in this organism. Inhibitors of ADPR-transferase activity also reduced the rate of differentiation of bloodstream forms of T.b.brucei to procyclic forms, suggesting the involvement of ADPR-transferase in the differentiation of T.b.brucei. However the inhibition of this enzyme did not effect the rate of proliferation.

CHAPTER 3

Materials and Methods

3.1 Materials

3.1.1 Rats

Female Wistar rats (≈ 250 g weight) were obtained from the Colony maintained at the animal house, Bath University.

3.1.2 Trypanosoma brucei

Trypanosoma brucei brucei (T.brucei, Strain MITat 1.1) were originally obtained from Dr. Paul Voorheis, Department of Biochemistry, Trinity College, Dublin 2, Ireland (Voorheis, H.P, 1977, Biochem. J. 164, 15-25). -

3.1.3 Reagents

All solvents and chemicals used in the preparation of solutions and buffers were of the Analar quality and were obtained from BDH chemical Ltd, Poole, Dorset. MTT, PMS, ADH, NAD and cis-aconitate were obtained from Sigmia Chemical Co. (London) Poole, Dorset. Dimethyl sulphate (DMS) was obtained from the Aldrich Chemical Co., Gillingham, Dorset. L-glutamine (200 mM), Penicilin/Streptomycin (5,000 I.U./ml and 5,000 mg/ml), Trypan blue (0.5% W/V in 0.85% W/V Salin) also supplied by Flow Laboratories. HEPES was obtained from Aldrich Chemicla Co.

3.1.4 Buffers

a) Krebs-Ringer phosphate buffer

Isotonic Krebs-Ringer phosphate buffer contained potassium dihydrogen orthophosphate (22 mM), magnesium sulphate (1 mM), sodium chloride (98 mM) and potassium chloride (2 mM). The pH was adjusted to pH 8.0 by using Sodium hydroxide (NaOH).

b) Triethanolamine buffer (TEA)

TEA buffer contained nicotin amide (100 mM), triethanolamine (100mM) and ethanol (0.5 M). The pH was adjusted to pH 7.4 with conc. hydrochloric acid (HCL).

c) Phosphate buffer saline (PBS)

PBS (Dulbecco's formula) tablets were obtained from Flow Laboratories, Irvin, Scotland.

3.1.5 Sucrose solutions

All Sucrose solutions (15-30% W/V) contained Sodium chloride (2.0 M), EDTA (10.0 mM), Tris-HCL (10 mM) and ethidium bromide (EtBr) 30 µg/ml. pH of the solutions was adjusted to pH 8.0 by using 1.0M NaOH.

3.1.6 Trypanosome lysis solution

Lysis solution contained sodium chloride (2.0 m), EDTA (10mM), Tris-HCL (100mM), Triton X-100 (0.5% V/V) and ethidium bromide 30 ug/ml. The pH of this solution was adjusted to pH 8.0 by using 1.0M NaOH.

3.1.7 Permeabilisation solutions

a) Hypotonic solution

Hypotonic solution contained HEPES (9 mM) pH 7.8, DTT (5 mM), Dextran (4.5% W/V), magnesium chloride (4.5 mM) and glucose (1% W/V).

b) Hypertonic solution)

Hypertonic soultion contained HEPES pH 7.8 (200 mM), Potassium

chloride (750 mM), DTT (5 mM), Dextran (4.5% W/V), EGTA (7.0 mM), Magnesium chloride (4.5 mM) and glucose (1% W/V).

3.1.8 Feeder cells

a) Baby Hamster Kidney cells (BHK 21)

Baby hamster kidney cells (BHK 21) were obtained from Flow Laboratories, Irvin, Scotland.

b) Rat spinal cord cell cultures

Rat spinal cord cell cultures were established from 15 day old rat embryos as described by Digby et al. (1985), these cultures were supplied by Dr. A.Jehanli at this Department (see methods 3.2.1.3).

c) Rat muscle fibroblast cultures

Rat muscle fibroblast cultures were established from 1 day old newborn rats and were supplied by Dr. A.Jehanli at this Department (see methods 3.2.1.4).

d) Fisher rat embryonic fibroblasts

Fisher rat embryonic fibroblasts were donated from Dr. F.Farzaneh, Molecular Genetics Unit, Kings college School of Medicine and Dentistry, Denmark Hill, London.

3.1.9 Culture media

a) RPMI 1640 medium

RPMI 1640 medium was purchased from Flow Laboratories at 10x concentration and made up to normal strength with HEPES solution pH 7.4 (20 mM). This medium was supplemented with 10% (V/V) serum (either calf, horse, fetal calf, or rabbit), penicilin (200 I.U./l),

Streptomycin (200 µg/l), glutamine (10 mM) and glucose 1% (W/V).

b) Dulbecco's Modification of Eagle's Minimal Essential Medium
(DMEM)

DMEM was obtained from Gibco at 1 x concentration. This medium was supplemented with 10% (V/V) serum (either calf, horse, fetal calf or rabbit), penicilin (200 I.U./l), Streptomycin (200 µg/l), glutamine (10 mM) and glucose (1% W/V).

c) Minimum Essential Medium (MEM)

MEM was obtained from Flow laboratories at 10x concentration and made up to normal strength with HEPES solution pH 7.4 (25 mM). This medium was supplemented with 10% (V/V) calf serum, penicilin (200 I.U./l), Streptomycin (200 µg/l) and glutamine (10 mM).

d) Medium SDM-79

SDM 79 medium was prepared according to the method of Brun and Schonenberger (1979).

<u>Ingredients</u>	<u>Per 1L</u>
Dulbecco's modified Eagle medium powder	7.0g
Medium 199 Powder	2.0g
MEM amino acid soln.	8.0ml
MEM non-essential amino acid soln.	6.0ml
Glucose (anhydrous)	1.0g
HEPES (BDH)	8.0g
MOPS	5.0g
NaHCO ₃	2.0g

<u>Ingredients</u>	<u>Per 1L</u>
L-Alanine	0.2g
L-Arginine	0.1g
L-Methionine	0.07g
L-Phenylalanine	0.08g
L-Proline	0.6g
L-Serine	0.06g
Taurine	0.16g
L-Threonine	0.35g
L-Tyrosine	0.1g
Adenosine	10.0mg
Guanosine	10.0mg
D(+) Glucosamine HCl	50.0mg
Folic acid	4.0mg
p-Aminobenzoic acid	2.0mg
Biotin	0.2mg
Penicilin G soln 5mg/ml	12.0ml
Gentamycin	20.mg

The pH of this medium was adjusted with NaOH (4M) to pH 7.3 and was filter sterilised (Millipore 0.2 µm). 2ml of filter sterilized bovine haemin solution (2.5 mg/ml in 50mM NaOH) was then added. This medium was supplemented with 10% (V/V) sterile heat inactivated fetal calf serum.

3.1.10 Sera

a) Horse serum

Heat inactivated donor horse serum was obtained from Flow laboratories, Irvin, Scotland.

b) Calf serum

Heat inactivated calf serum was obtained from Flow laboratories, Irvin, Scotland.

c) Fetal calf serum

Heat inactivated fetal calf serum was obtained from Gibco.

d) Rabbit serum

Heat inactivated commercial rabbit serum was obtained from Flow Laboratories, Irvin, Scotland. Fresh rabbit serum was prepared using the method of Baltz, T., et al., (1985), (see methods 3.2.1.2).

3.1.11 Anion-Exchanger (DEAE-Cellulose)

Whatman pre-swollen microgranular anion-exchanger, Diethyl amino ethyl cellulose, (Type DE-52) was obtained from Whatman, Maidstone, Kent, U.K.

3.1.12 Inhibitors

3-aminobenzamide, 3-acetamidobenzamide, 3-acetamidobenzoic acid were synthesized in this laboratory by Mrs B.Hunt. 3-aminobenzoic acid was purchased from Aldrich Chemical Company Ltd., Gillingham, Dorset.

3.1.13 Radioactive Ligands and Scintillation Counting

[³H]-NAD was prepared from [³H]-ATP (Radiochemical centre) by Dr. W.J.D. Whish according to the method of Ohtsu and Nishizuka (1971). [³H]-NAD was prepared in ethanol with a final specific

activity of 20 mci/ μ mole and radioactive concentration of 1 mci/ml. 3 MM (24 mm) filter paper discs and glass fibre discs (GF/C) were supplied by Whatman Ltd, Maidstone, Kent, U.K.

3.2 Methods

3.2.1 Cell Culture Methods

3.2.1.1 Preparation of Trypsin

1% (W/V) trypsin was prepared by dissolving 100 mg of trypsin in 10 ml of cold PBS. 0.1 ml aliquotes of this solution were dispensed into sterile storage tubes as quickly as possible, as the trypsin will begin digesting itself, and stored at -20°C.

3.2.1.2 Preparation of Rabbit Serum

Rabbit serum was freshly prepared from blood taken by heart puncture of adult animal. After coagulation at room temperature for 3-4 hours, the blood was stored overnight at 4°C. The serum then was centrifuged for 15 minutes at 4000 g, complement inactivated by incubation at 56°C for 30 minutes, filter sterilised (Millipore 0.2 μ m) and stored in 10 ml portions at -20°C.

3.2.1.3 Preparation of Rat Spinal Cord Cell Cultures

Rat spinal cord cell cultures were established as described by Digby et al. (1985). Spinal cords (13-15) from 14-16 day old rat embryos were minced in 0.5 ml of Puck's DIG balanced salt solution (Puck's BSS). Puck's BSS contained Puck's DI salt solution (50 ml, 20 fold concentration), glucose (6 g/liter), sucrose (15 g/liter), in 10 mM HEPES buffer, pH 7.3. This was transferred to a 12 ml test tube

(Sterilin, Middlesex, England) in the same buffer (4 ml). Deoxyribonuclease II (1 mg/ml) in H-Eagle's medium (200 μ l) was added, followed after 5 minutes at 20°C by 0.5% trypsin (200 μ l) (bovine pancreas, type III) in H-Eagle's medium. The cells were incubated at 37°C for 45 minutes, with gentle mixing at 5-minute intervals, after which soya bean trypsin inhibitor (200 μ g) in cold medium A (DMEM supplemented with 20% (V/V) fetal calf serum, glucose (6 g/liter) and 2 mM glutamin) was added and the mixture was centrifuged for 5 minutes at 400 g. The supernatant was removed, and the tissue fragments were mechanically dissociated by trituration with a plastic Pasteur pipette (Sterilin) using aliquotes (3 ml) of medium A. The cell suspension ($3-5 \times 10^6$ cells/ml) was plated onto Petri dishes at a density of 10^5 cells/cm² in culture medium (2.5 ml or 0.75 ml for 3.5 cm² and 1.6 cm² Petri dishes, respectively). Cultures were maintained in a humidified atmosphere containing 10% CO₂ at 37°C. Cells were plated in medium A and thereafter the medium was changed every 3 days. The cells were used after 3 weeks in culture.

3.2.1.4 Preparation of Rat Muscle Fibroblast Cultures

Rat muscle fibroblast cultures were established from 1 day old newborn rats as described below:

Hind leg muscle was dissected and minced in Puck's BSS by using iridectomy scissors. The tissue was further dissociated by enzymatic digestion for 1 hour at 37°C with trypsin (1.25 mg/ml) and collagenase (5 mg/ml). The suspension was centrifuged and single cells released from the tissue fragments by trituration with a plastic Pasteur pipette (Sterilin) in medium. The cells were seeded in medium in 3.5 cm² Petri dishes (3×10^5 cells/dish, in 4 ml medium).

The medium was changed every 3 days. The cultures were used after 8 days in vitro. By this time they consisted mainly of fibroblasts and some myotubes.

3.2.1.5 Maintenance of Tissue Culture Cell Lines

The overlays of confluent feeder cells were decanted and the monolayers were washed with PBS to remove serum (trypsin inhibitors). PBS was then decanted and 2 ml of 0.05% trypsin, (diluted from a 1.0% stock solution in MEM) was added to each T-25 flask. These were incubated at 37°C until the monolayers were detached and the cells were rounded up (normally 5-6 minutes). At this time 2 ml of culture medium containing 10% serum was added to each T-25 flask to inactivate the trypsin. A small volume of cell suspension was mixed with an equal volume of a solution of 0.2% (W/V) trypan blue in physiological saline (0.9% NaCl). Cell viability was estimated by determining the fraction of cells permeable to trypan blue. In trypan blue, the nuclei of permeable cells stained blue. Cells were diluted with 10 ml of the culture medium, in T-25 flask, to obtain a cell density of approximately 1×10^4 cells/ml. Cultures were incubated in a humidified 5% CO₂/air atmosphere at 37°C.

3.2.1.6 Storage of the Cell Lines

The suspension of cells obtained by trypsinisation of sub-confluent feeder-cells were cooled in an ice bucket. Dimethyl sulphoxide (DMSO) was added to a final concentration of 10% (V/V) to this cell suspension and mixed well. Then 1 ml aliquotes were dispensed into Nunc nitrogen storage tubes. These were loaded into a biological freezer (Union Carbide BF-6) and lowered into the vapour phase of a liquid nitrogen storage tank for 2 hours (a maximum of

eight tubes could be loaded into the biological freezer at one time). The frozen cells were then transferred to a liquid nitrogen storage tank, where they were kept until required.

3.2.1.7 Preparation of Trypanosomes

A strain of T.brucei, MITat 1.1, (stored in a sterile rat blood stabilate in liquid nitrogen until use) was used to infect female Wistar rats (\simeq 250g weight) by intraperitoneal injection. Trypanosomes were prepared for infection of rats in isotonic Krebs-Ringer phosphate buffer (see materials 3.1.4a) and injected into rats at a dosage of 10^7 viable trypanosomes per animal. The viability of the trypanosomes was monitored by using phase contrast microscopy, and active mobile trypanosomes were counted by using a haemocytometer.

3.2.1.8 Purification of Bloodstream forms of T.brucei

At about 71 hours post infection, at the peak of the parasitemia, $>10^9$ trypanosomes/ml of blood, the inoculated rats were bled. Heparin (200 units/ml) was used as anticoagulant. The parasites were separated from the blood by density centrifugation at 350 g for 10 minutes. Trypanosomes removed from the interface layer, were washed and resuspended in Krebs-Ringer phosphate buffer (see materials 3.1.4a) containing glucose (1% W/V) and sucrose (3% W/V). The washed trypanosomes were purified by using the method described by Lanham (1968) and Lanham & Godfrey (1970). This purification procedure is based on a difference of surface charge between host blood cells and trypanosomes. Blood cells carry a more negative charge than do trypanosomes. Therefore, it is possible, under certain conditions of pH (8.0) and ionic strength to adsorb the

negatively charged anion exchanger (DEAE-cellulose) while the trypanosomes pass through, retaining viability and infectivity.

3.2.1.9 In vitro Cultivation of Bloodstream forms of T.brucei

The culture system consisted of a mammalian feeder layer in tissue culture medium containing 10% serum (either calf, horse, fetal calf or rabbit). 2-4 days old near-confluent feeder-cell cultures were used. Shortly before inoculation, the overlay from these cultures was discarded and replaced with fresh medium. The experiments were carried out in T-25 flasks. Each culture was inoculated with 1×10^4 freshly isolated trypanosomes/ml of medium. The cultures were then incubated in a humidified incubator at 37°C in 5% CO₂ and 95% air. Every 24 hour the trypanosome density was estimated.

3.2.1.10 Preparation of Cis aconitate

A stock solution of 30 mM cis-aconitate was made up in distilled water and the pH was adjusted with NaOH (5M) to pH 7.4. This solution was filter sterilised (Millipore 0.2 µm) and stored at 4°C.

3.2.2 Biochemical Methods

3.2.2.1 Estimation of NAD

NAD was assayed essentially as described by Nisselbaum and Green (1969), the mechanism of the reaction is shown in Fig. (3.1). In this method, NAD containing samples were added to a suitably buffered mixture. NAD is reduced to NADH by alcohol dehydrogenase which in turn reduces the chromagen mixture MTT and PMS to a purple coloured formazan. The rate of increase in absorbance due to the purple

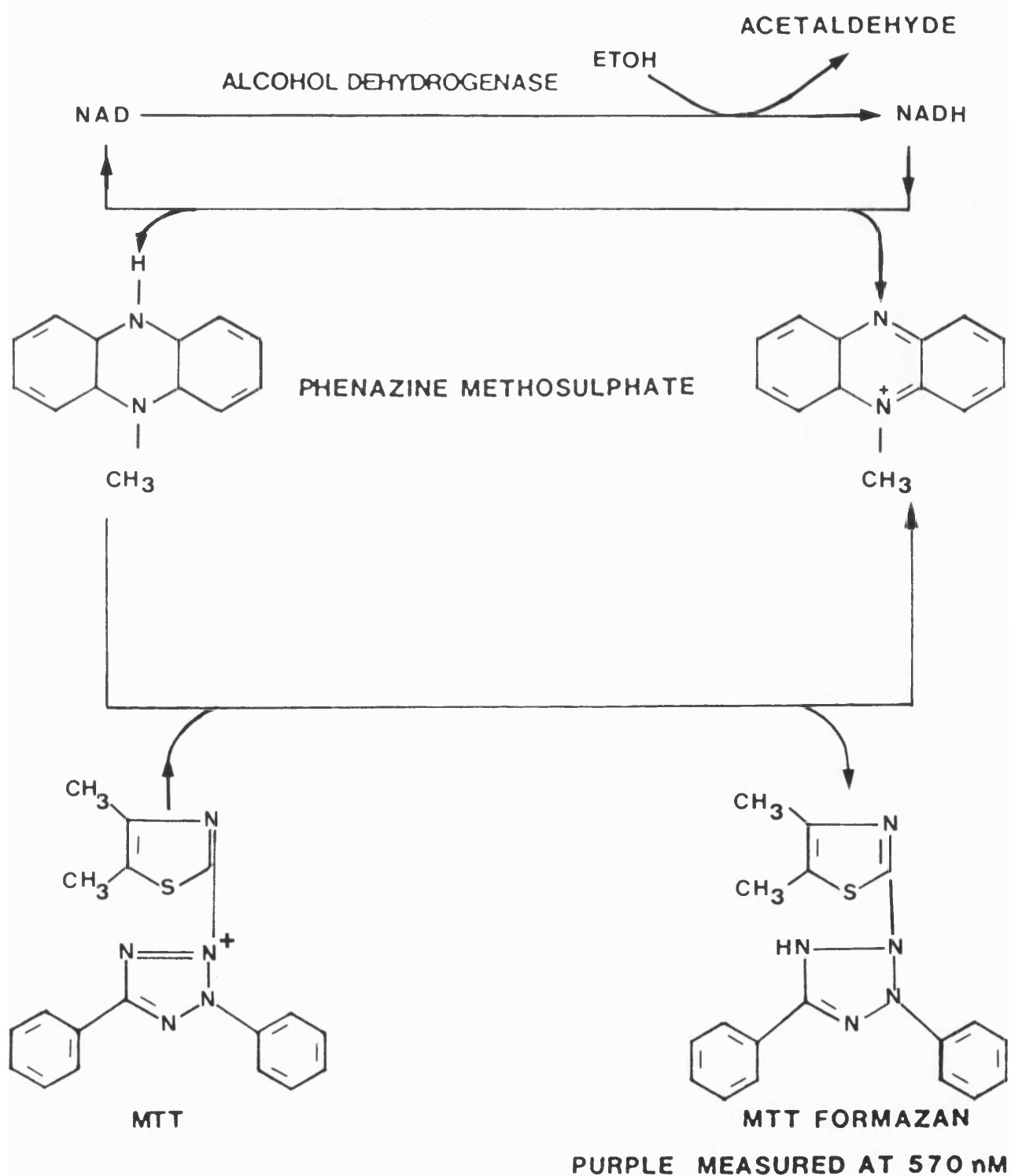


Fig. 1.3 The principle of Nisselbaum & Green assay for NAD

colour is dependent on the concentration of NAD + NADH and was measured by estimating the increase in absorbance at 560 nm. The reaction mixture contained 0.8 ml of PMS (1 mg/ml), 0.05 ml of MTT (5 mg/ml), 0.1 ml of ADH (1 mg/ml) and 1.95 ml of triethanolamine (TEA) buffer (for the composition of TEA buffer see materials 3.1.4b).

3.2.2.2 NAD Level in T.brucei

Trypanosomes were collected by centrifugation in a microcentrifuge for 30 seconds and the pelleted cells broken in 50% ethanol and sonicated using a bath sonicator (DAWE SONICLEANER TYPE 6441A) for 15 minutes. The mixture was recentrifuged and 100 μ l of the supernatant was assayed for NAD content. Results were expressed as p mol NAD/ 10^6 trypanosomes.

3.2.2.3 Preparation of Anion-Exchanger (DEAE-Cellulose)

Whatman pre-swollen microgranular anion-exchanger (Type DE-52) was used. The DEAE-cellulose was equilibrated with Krebs-Ringer phosphate buffer (without glucose and sucrose) in the ratio of 100 g of pre-swollen adsorbant to 1.5 litre of buffer. The adsorbent was allowed to settle for about 20 minutes and the supernatant, containing the fines, was decanted off. This was repeated five to six times and finally the adsorbent was resuspended in Krebs-Ringer phosphate buffer (without glucose and sucrose), pH 8.0. The preparation of the column and the fractionation of the blood were both carried out at 4°C and the eluates were kept in ice baths.

3.2.2.4 Irradiation of Trypanosomes

Trypanosomes were irradiated in a Cobalt-60 source emitting

0.5K rads of γ -radiation per minute. The irradiation was performed at room temperature and trypanosomes were immediately placed on ice until used.

3.2.2.5 ADPR-transferase assay

In order to estimate the physiological ADPR-transferase activity 250 μ l aliquots of permeabilised trypanosomes in the hypotonic buffer, at a density of approximately 10^8 cells/ml, were transferred to a water bath at 26°C and the assay was started by the addition of 50 μ l hypertonic solution containing, 10 μ l [3 H]-NAD (20 mci/ μ mole, 1 mci/ml) and 10 μ l of 100 mM DTT. The final concentrations in the assay were: NAD 1.6 μ M, HEPES 38 mM (pH 7.8), KCl 117 mM, DTT 7.8 mM, EGTA 1 mM, $MgCl_2$ 4.2 mM, Glucose 1% and Dextran 4.2%. 20 μ l aliquots were withdrawn at the indicated time points and the acid insoluble radioactivity was estimated by liquid scintillation counting.

3.2.2.6 DNA Molecular Weight Studies Using Nucleoid Gradients

DNA molecular weight changes were studied by measuring the sedimentation rate of trypanosome nucleoids according to the method of Farzaneh et al. (1985). 100 μ l of trypanosome suspension (approximately 5×10^7 trypanosomes) was gently deposited in 300 μ l of lysis solution which has already been overlaid on 14 ml 15-30% linear sucrose gradient in cellulose nitrate tubes. (For composition of the sucrose solutions and lysis solution (see materials 3.1.5 & 3.1.6).

The gradients were kept in the dark for 30 minutes at room temperature and then centrifuged at 23K rpm (approximately $100 \times 10^3 g$) in a Beckman SW 27 rotor for 2 hours.

To identify the position of nucleoids bands; gradients containing ethidium bromide, were visualised with an ultraviolet

transilluminator and the position of nucleoids were marked. The distance travelled by the nucleoids was measured by the distance from the position of the nucleoids band in the gradient to the bottom of the layer at the top of the gradient. Relative distance were then estimated as the ratio of the distance travelled by damaged nucleoids to the distance travelled by undamaged (reference) nucleoids in the same centrifugation.

3.2.2.7 Trypanosome Permeabilisation

Cells were permeabilised to NAD by an osmotic shock procedure (Farzaneh et al., 1985). Washed trypanosomes were resuspended at a density of approximately 1×10^8 /ml in a hypotonic solution (see materials 3.1.7a). Trypanosomes were kept on ice, until more than 90% became permeable; then the solution was made isotonic by the addition of 1/5 Vol. of a hypertonic solution (see materials 3.1.7b). Permeabilisation was routinely achieved after about 15 minutes and was monitored by adding 1/5 Vol. of a solution of 0.2% (W/V) Trypan blue in the hypertonic buffer to a small sample of trypanosomes. The percentage of Trypan blue positive cells was estimated microscopically.

3.2.2.8 Measurement of Acid Insoluble Radioactivity

Acid-insoluble radioactivity was measured by adding an aliquot of the reaction mixture containing the acid insoluble radioactivity to approximately 1-2 ml of ice-cold 20% (W/V) trichloroacetic acid (TCA). The samples were then filtered onto Millipore GF/C discs (prewashed with 3 ml ice-cold 5% TCA) using a Millipore ultra filtration tower. The discs were washed three times with 5 ml of ice-cold 5% TCA and once with 5 ml of 95% ethanol and then left to

dry at room temperature for 2 hours or in an oven at 60°C for 10-15 minutes.

3.2.2.9 Scintillation Counting

All radioactive samples were counted in a Packard Tri-Carb liquid scintillation spectrometer. The scintillant used was 0.5% (W/V) PPO, 30% (V/V) Triton-x100, 70% (V/V) Toluene (scintillant for aqueous samples). Usually 3 mls of scintillant was used. The GF/C filter discs used were always placed face uppermost in the scintillation vials. Counting efficiency was 28% for [^3H]-NAD.

CHAPTER FOUR

DNA Damage and NAD metabolism in T.brucei

Results and Discussion

4.1 Introduction

It has previously been demonstrated that treatment of cells with DNA damaging agents results in a rapid lowering of the NAD levels with a concomitant increase in ADPR-transferase activity (Whish et al., 1975; Skidmore et al., 1979; Durkacz et al., 1980). The increase in ADPR-transferase activity may be brought about by either alkylating agents, irradiation or endonucleases. The common effect of all these agents is that they ultimately result in DNA strand-breaks. The correlation between the drop in NAD and the increased ADPR-transferase activity, in a dose dependent manner (as observed by Durkacz et al., 1980), suggests that ADP-ribosylation may have a role in DNA repair.

In this section the aim was to study the effect of DMS and γ -radiation on the cellular NAD level in T.brucei. DMS, a monofunctional alkylating agent used for investigation here, is an electrophilic compound (Miller, 1970) which reacts with nucleophilic sites in the DNA, particularly the N₇ of guanine and to a lesser extent, the N₇ of adenine (Lawley, 1966). The introduction of the methyl group at the N₇ position of guanine destabilizes the glycosidic linkage between the N₉ of purine and C₁ of deoxyribose, thus making the bond more susceptible to hydrolysis. Although the N₇ of guanine is not involved in hydrogen bonding in this position it does lead to the production of apurinic sites. This leads to the subsequent formation of single strand-breaks in the polynucleotide backbone. Thus damage to DNA will result in helix distortion and the loss of function in biological processes such as replication, transcription and cellular differentiation. However, cells possess the capacity to cope with limited damage to their DNA in the form of

repair processes such that the damaged regions are enzymatically removed from DNA and replaced by a new sequence of intact nucleotides; closing the gap by ligase action. For recent reviews of ADPR-transferase involvement in cellular processes see (Althaus, Hilz & Shall 1985 and Gaal & Pearson 1985, 1986).

4.2 Results

4.2.1 In vitro Maintenance of T.brucei

Trypanosomes (*brucei brucei*) were maintained in RPMI 1640 medium (x10), made up to normal strength with HEPES solution (20 mM) pH 7.4, in the absence and presence of 2mM 3AAB at 37°C. This medium was supplemented with 0.15% (w/v) bovine serum albumin together with the components outlined in the materials section. Microscopic examination of T.b.brucei suggested that at 37°C, in the presence or absence of 2mM 3AAB, these organisms remain motile and therefore presumably alive for at least 3 hours in the above medium. Fig. (4.1) demonstrates that the in vitro system for the maintenance of the trypanosomes used in these studies is adequate for at least up to 3 hours since there is no reduction in the motility of the trypanosomes during this period. However soon after this period there is a rapid decline in the viability of the trypanosomes. This was shown to be independent of alterations in the pH. Fig. (4.2) demonstrates that the pH of the culture is stable over this period and that the 0.6 pH unit drop had no effect on the viability of trypanosomes. The sharp decrease in the number of motile trypanosomes was also found to be independent of glucose depletion since the addition of extra 1% glucose at 90 and 150 minutes when there is still little or no reduction in the mortality of the trypanosomes has no effect on subsequent rate of decline in viability.

4.2.2 Estimation of NAD level in T.brucei

In order to estimate the NAD level in T.brucei a standard curve was established (Fig. 4.3). NAD was assayed essentially as described by Nisselbaum and Green (1969) (see methods 3.2.2.1). Trypanosomes

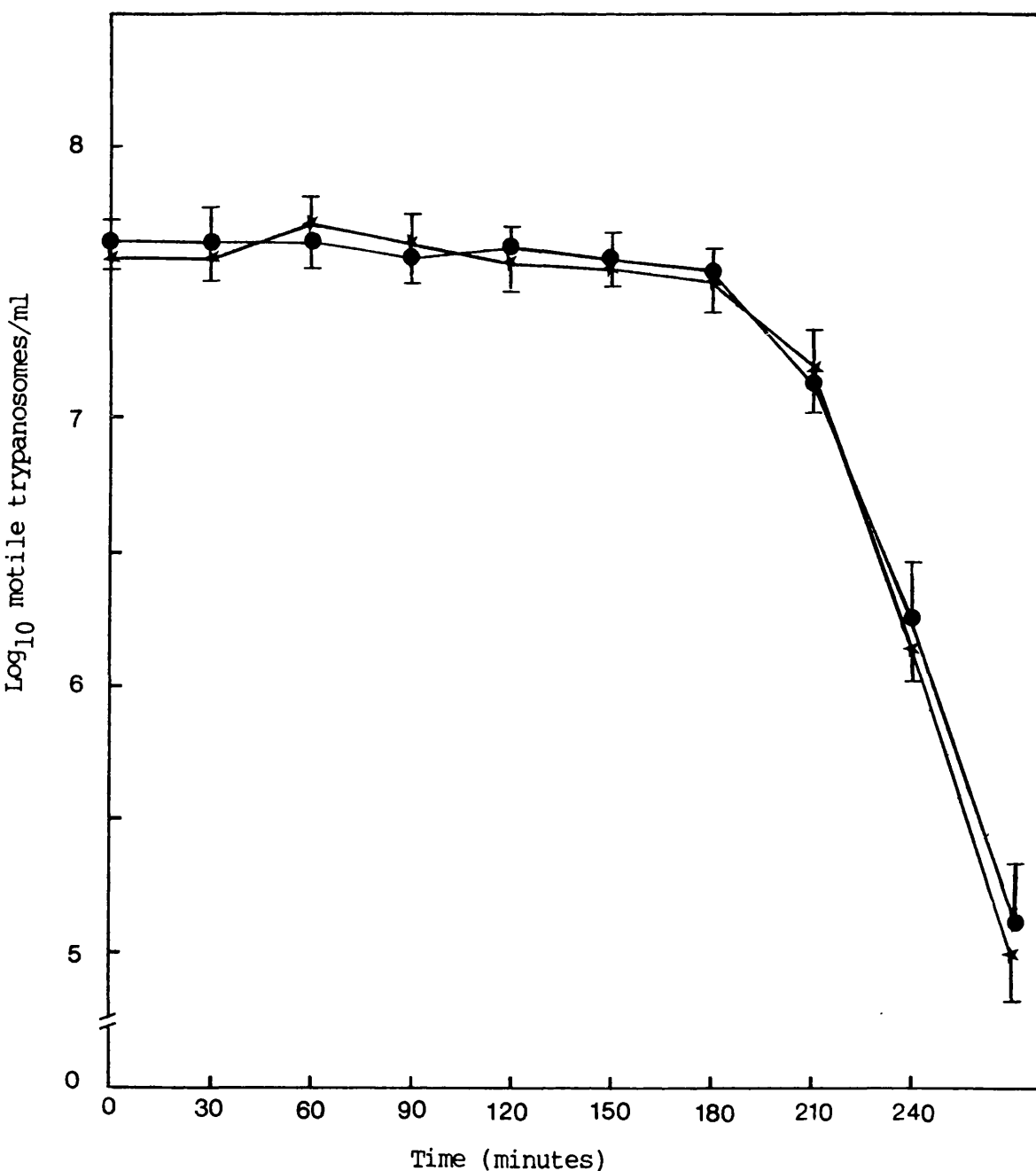


Fig. 4.1

In vitro maintenance of *T. brucei*. Trypanosomes were incubated in HEPES buffered RPMI at 37°C in the presence and absence of 3AAB (2mM). Trypanosomes' motility was monitored by using phase contrast microscopy, and active trypanosomes were counted using a haemocytometer. Control cells (●), cells treated with 2mM 3AAB (★). Each point is the mean of 2 separate experiments in each of which trypanosomes were counted in duplicates, the bars indicate the standard error of the mean.

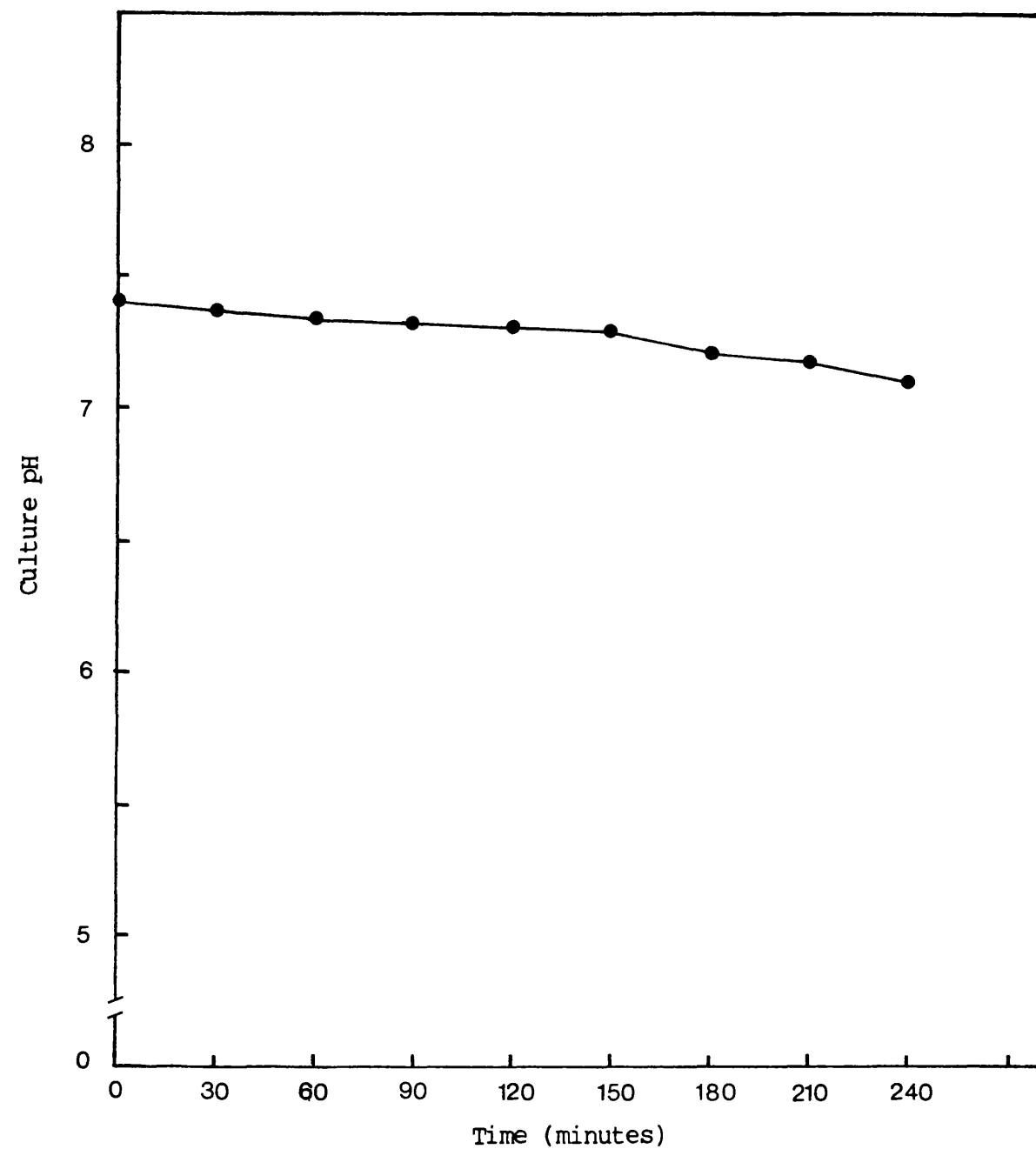
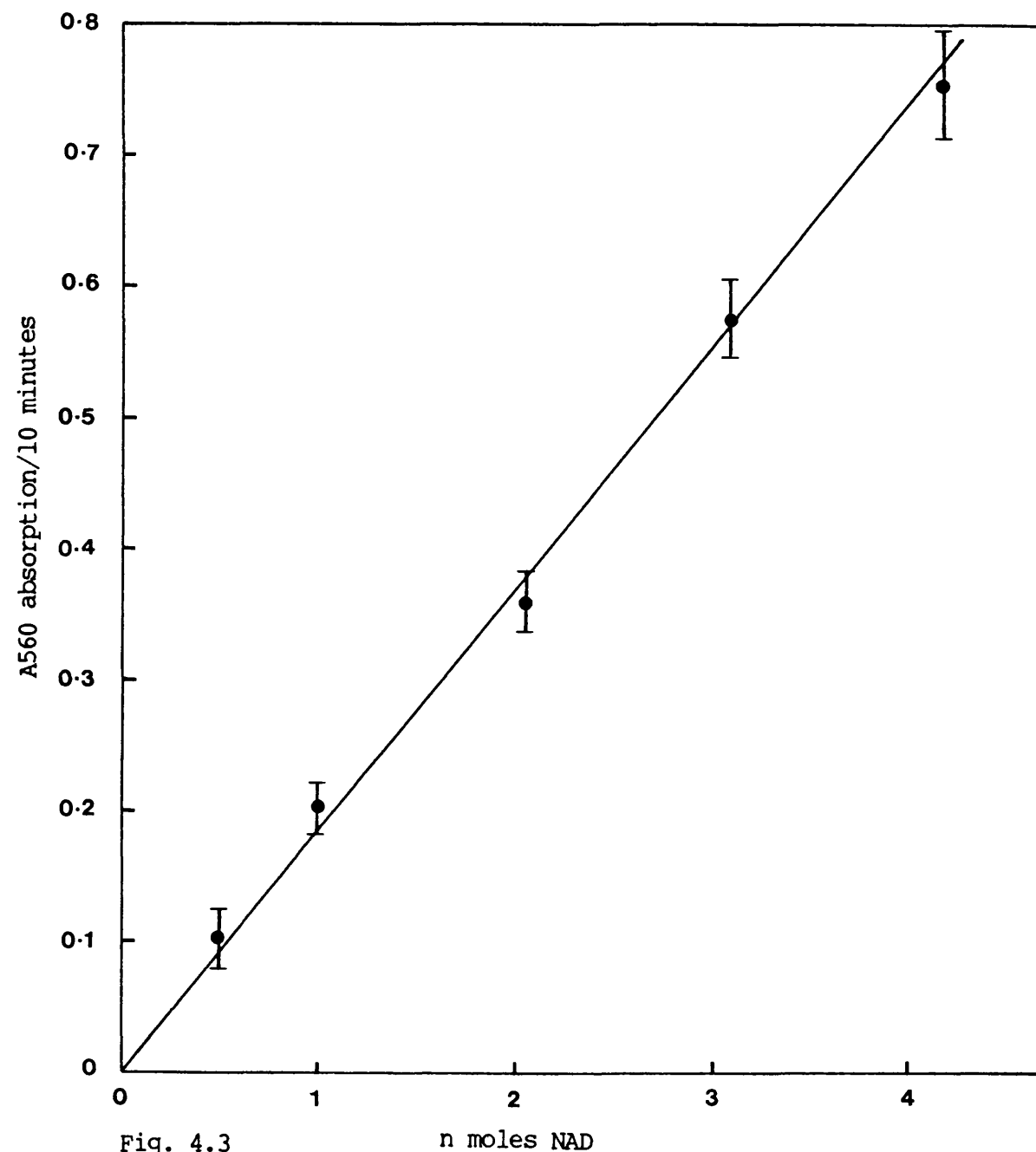


Fig. 4.2

pH alteration in T.brucei culture media. Trypanosomes were incubated in HEPES buffered RPMI at 37°C. The pH of the medium was measured every 30 minutes.



NAD standard curve. NAD level in T.brucei was estimated according to the method of Nisselbaum & Green (1969). This involved measurement of the NAD level in standard solutions. Based on similar measurements, the NAD level in T.brucei was found to be 13.2 ± 2 pmol NAD/ 10^6 trypanosomes. Each point on the standard curve is also the mean of six independent measurements, the bars indicate the standard error of the mean.

were used at a density of 5×10^7 cells/ml in HEPES buffered RPMI medium supplemented with 0.15% (W/V) bovine serum albumin. The NAD level in T.brucei was found to be 13.2 ± 2.5 pmol NAD/ 10^6 trypanosomes (n=10). Based on the published figures for the DNA content in trypanosomes (0.097 pg nucleus, 0.004 kinetoplast) the NAD content can be calculated as approximately 132 pmoles per mg DNA.

4.2.3 The Response of T.brucei to DMS

DMS was made up in 50% (V/V) ethanol/water (final ethanol concentration, 0.25% (V/V)). To 10ml aliquots of cells, (5×10^7 trypanosomes/ml) in the HEPES buffered RPMI medium DMS was added to a variety of final concentrations ranging from 10 μ M to 1 mM. The resulting cultures were mixed well and incubated at 37°C in a water bath. Samples (1 ml) were removed at each time point, microcentrifuged for 30 seconds, the cell pellet was resuspended in 50% ethanol and sonicated using a bath sonicator (DAWE SONICLEANER TYE 6441A) for 15 minutes. The resulting mixture was recentrifuged and the supernatant assayed for NAD content (see methods 3.2.2.1).

At 1 mM DMS (Fig. 4.4), there was no effect on the cellular NAD level for 90 minutes. After this time the NAD level fell in a linear fashion over a period of 30 minutes to 50% of the initial level at which it remained for nearly an hour. When trypanosomes were treated with 0.5 mM DMS (Fig. 4.5), the NAD level did not drop during the first 70 minutes, after which time the level dropped rapidly. The NAD drop occurred over a subsequent period of 70 minutes, falling from 13.2 ± 2.5 pmol/ 10^6 cells to 2.6 ± 1 pmol/ 10^6 cells at which it remained for at least 30 minutes. During all of these time courses greater than 95% motility was maintained; however, after 3 hours the cells rapidly died.

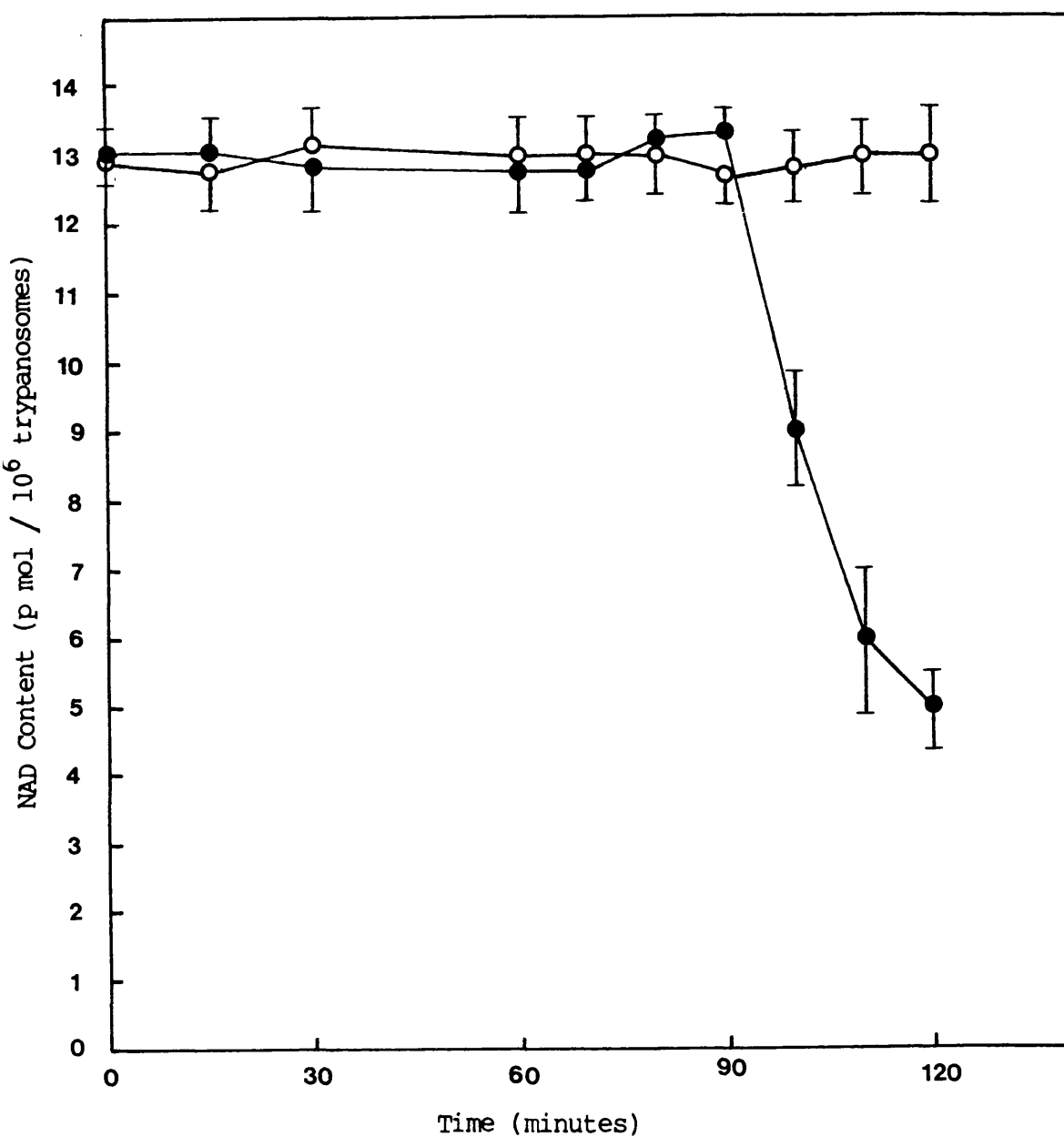


Fig. 4.4

The effect of continuous 1.0mM DMS treatment on the cellular NAD content. The NAD level was estimated by the method of Nisselbaum and Green (1969). The NAD content is expressed as p mol NAD/10⁶ trypanosomes. Control cells (O), 1.0mM DMS (●). Each point is the mean of 6 measurements in 3 separate experiments, the bars indicate the standard error of the mean.

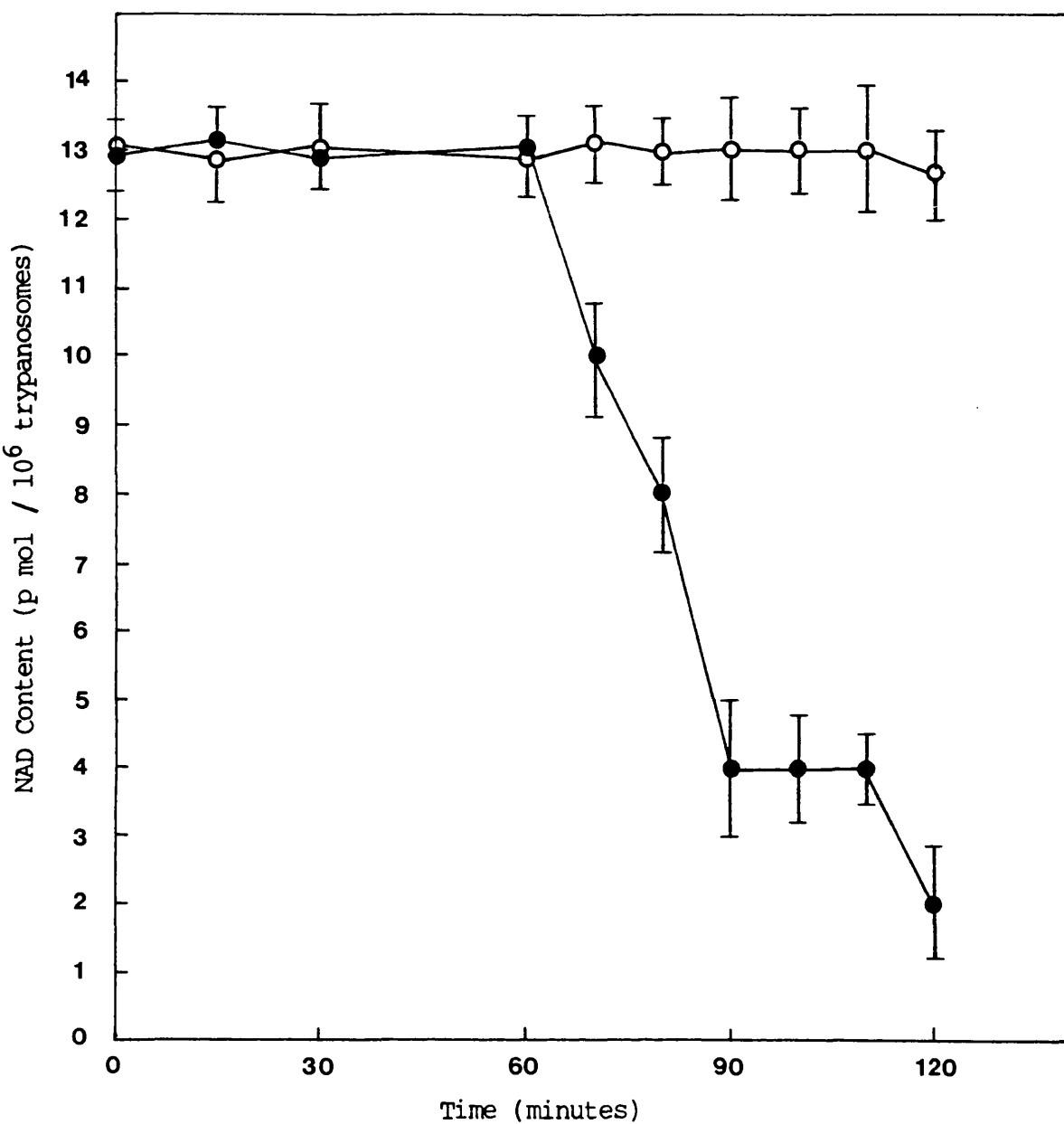


Fig. 4.5

The effect of continuous 0.5mM DMS treatment on the cellular NAD content. The NAD level was estimated by the method of Nisselbaum and Green (1969). The NAD content is expressed as pmol NAD/10⁶ trypanosomes. Control cells (O), 0.5mM DMS (●). Each point is the mean of 6 measurements in 3 separate experiments, the bars indicate the standard error of the mean.

The lag phase of 70-90 minutes was always seen in both 0.5 mM and 1 mM DMS treatment respectively. This suggested that in fact the DMS concentrations used so far may have been too high possibly causing the inactivation of the NAD metabolising enzymes thus preventing or slowing the NAD loss. To investigate this possibility trypanosomes were treated using lower concentrations of DMS, that is 10 μ M to 300 μ M. As can be seen in (Fig. 4.6), the cellular NAD level dropped without a lag phase at all of these lower DMS concentrations. The drop in NAD level seems to be linear over DMS concentrations up to 100 μ M and gradually decreases at higher concentrations (Fig. 4.7). Table (4.1) presents a summary of the data in (Fig. 4.6). It shows that the initial rate of NAD drop is proportional to the DMS concentration. However when trypanosomes are treated with 0.5 mM and 1 mM DMS this drop is delayed with a lag phase of some 70-90 minutes. To investigate the possibility of ADPR-transferase inactivation by high concentration of DMS, physiological and total ADPR-transferase activities were measured as described in the next section.

4.2.4 ADPR-transferase Assay

Trypanosomes are impermeable to NAD, the specific substrate for ADPR-transferase. In cells permeabilised to NAD by a mild hypotonic shock (see methods for experimental details 3.2.2.6) two levels of ADPR-transferase activity can be measured:

- i) The basal or physiological activity, in the absence of experimentally induced DNA strand-breaks.
- ii) The potential or maximum activity displayed in the presence of experimentally induced DNA strand-breaks, formed by the action of exogenously supplied DNA damaging agents.

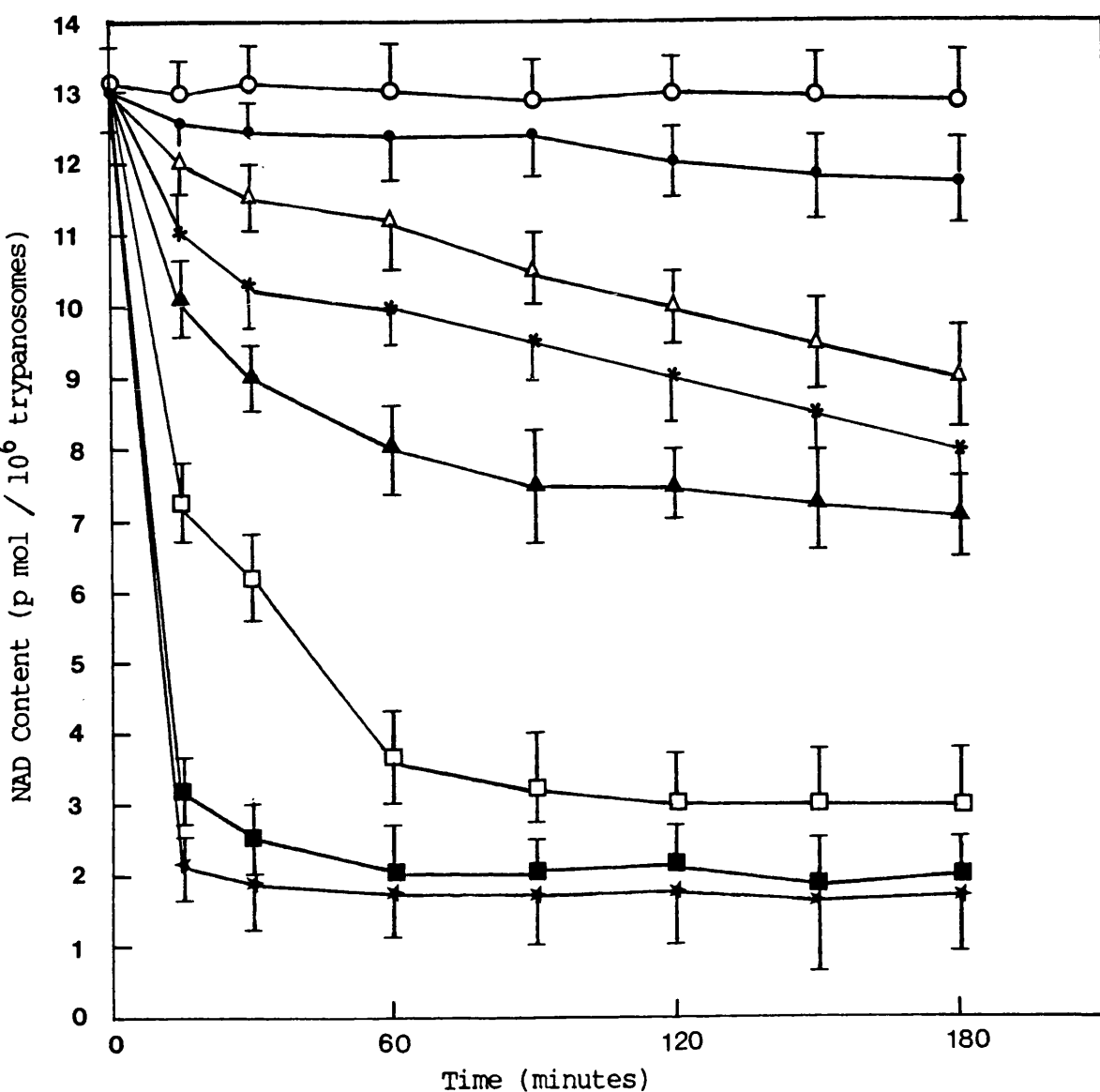


Fig. 4.6

The effect of continuous treatment with various doses of DMS on cellular NAD content. The NAD content was measured after treatment of trypanosomes with various doses of DMS at 37°C. Resulting NAD levels were estimated by the method of Nisselbaum & Green (1969). NAD content is expressed as pmol NAD/10⁶ trypanosomes. Control cells (O), 10 μM DMS (●), 20 μM DMS (Δ), 30 μM DMS (*), 50 μM (▲) DMS, 100 μM (□) DMS, 200 μM (■) DMS and 300 μM DMS(★). Each point is the mean of 6 measurements in 3 separate experiments, the bars indicate the standard error of the mean.

Table 4.1 Relationship Between DMS Concentration
and the Cellular NAD Drop

DMS (μM)	The initial rate of NAD Degradation p mole NAD/min/ 10^6 trypanosomes
Control	0.0
10	0.03
20	0.073
30	0.14
50	0.18
100	0.39
200	0.65
300	0.73

The initial NAD degradation rate was determined using the 15 minutes linear portion of NAD degradation presented in (Fig. 4.6).

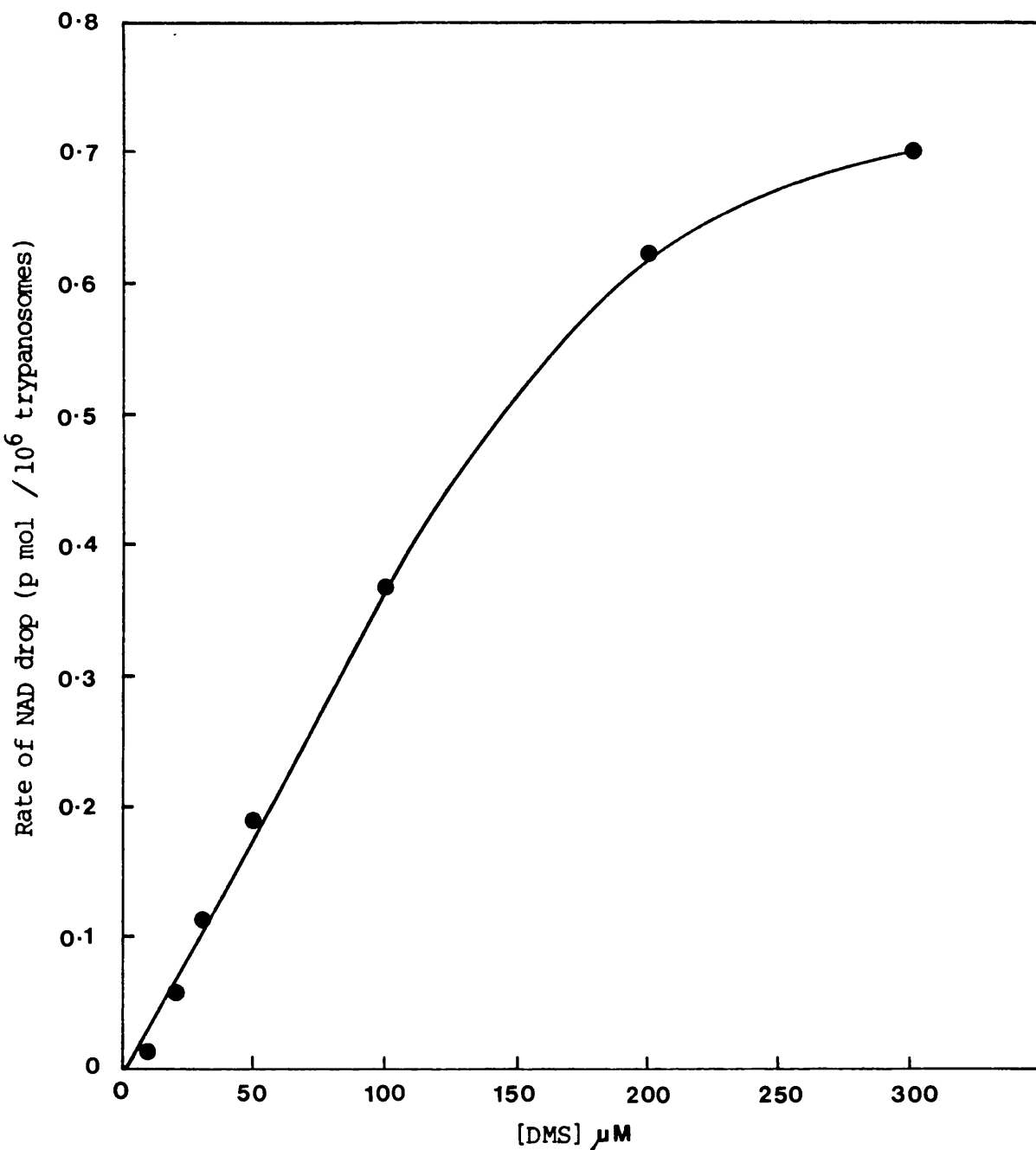


Fig 4.7

Relationship between the drop in cellular NAD level and DMS concentration. The NAD content was measured after continuous treatment of trypanosomes with various doses of DMS at 37°C. The rate of NAD drop in the DMS treated trypanosomes was estimated from the data in (Fig. 4.6) using the initial linear 15 mins. The rate of degradation is expressed as pmol NAD/min/ 10^6 trypanosomes.

In order to estimate the physiological ADPR-transferase activity 250 μ l aliquots of permeabilised trypanosomes in the hypotonic buffer, at a density of approximately 10^8 cells/ml, were transferred to a water bath at 26°C and the assay was started by the addition of 50 μ l hypertonic solution containing, 10 μ l [3 H]-NAD (20 mci/ μ mole, 1 mci/ml) and 10 μ l of 100 mM DTT. The final concentrations in the assay were: NAD 1.6 μ M, HEPES 38 mM (pH 7.8), KCl 117 mM, DTT 7.8 mM, EGTA 1 mM, MgCl₂ 4.2 mM, Glucose 1% and Dextran 4.2%. 20 μ l aliquots were withdrawn at the indicated time points and the acid insoluble radioactivity was estimated by liquid scintillation counting (see methods 3.2.2.7). Permeabilisation was routinely achieved after about 15 minutes of exposure to the hypotonic buffer when more than 90% of trypanosomes became permeable (see methods 3.2.2.6).

For the estimation of the potential ADPR-transferase activity trypanosomes were treated with 0.1 mM, 0.5 mM and 1 mM DMS for 30 minutes at 37°C prior to permeabilisation. The potential ADPR-transferase activity was then measured as described for the physiological activity.

In T.brucei permeable to NAD there is a low but measurable physiological ADPR-transferase activity (Fig 4.8, ▲). Induction of DNA strand-breaks increases this activity. This is clearly evident in trypanosomes which are treated with 0.1 mM DMS for 30 minutes at 37°C prior to permeabilisation (Fig 4.8, ●). In these cells the ADPR-transferase activity is increased about 3.5 fold over the physiological ADPR-transferase activity.

Consistent with the suggestion that high concentrations of DMS were inactivating ADPR-transferase activity in L1210 cells (Al-Muhtaseb, 1985) it was shown that DMS treatment at 1 mM (✱) and

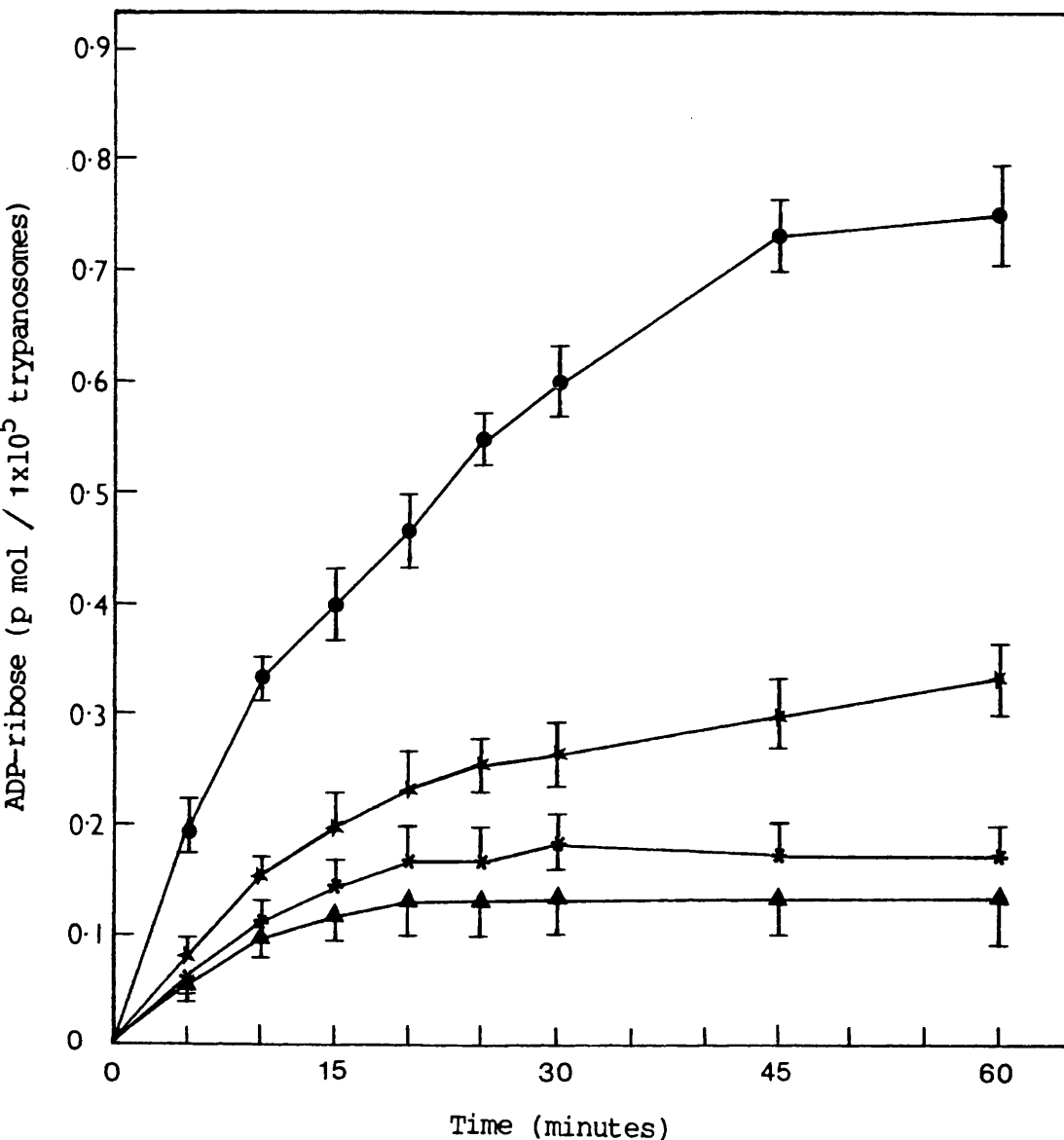


Fig 4.8

ADPR-transferase activity in *T.brucei* permeabilised to NAD by hypotonic shock. Trypanosomes, freshly isolated from an infected female Wistar rat, were made permeable to [3 H]-NAD (see methods 3.2.2.6). Physiological and potential ADPR-transferase activity were measured in the presence of $1.6 \mu\text{M}$ [3 H]-NAD (specific activity 20 mci/m mole and radioactive concentration of 1 mci/ml). For the estimation of the potential ADPR-transferase activity trypanosomes were treated with 0.1 mM DMS, 0.5 mM and 1 mM at 37°C for 30 min. prior to permeabilisation. Physiological activity (▲), potential activity; 0.1 mM (●), 0.5 mM (*) and 1 mM (*) DMS. The acid insoluble radioactivity was then estimated by liquid scintillation counting. Each point is the mean of 4 measurements in 2 separate experiments, the bars indicate the standard error of the mean.

0.5 mM (★) resulted in a lower increase in ADPR-transferase activity compared to the activity of this enzyme treated with the lower concentration of 0.1 mM DMS (●) (Fig. 4.8). This finding is in disagreement with Durkacz et al., (1980) who found that ADPR-transferase activity was increased by 3-fold when L1210 cells were treated with 1 mM DMS. Al-Muhtaseb (1985) reported that the enzyme ADPR-transferase was inactivated in permeabilised Mouse L1210 cells after treatment with high concentrations of DMS. This report indicates 33.4% and 52% loss of enzyme activity after 0.5 mM and 1 mM DMS treatment. ThiMan & Shall (1982) also demonstrated that at DMS concentrations higher than 0.5 mM the synthesis of poly(ADP-ribose) declined. They reported that at these higher concentrations the cells were visibly shrunken when examined by phase-contrast microscopy.

4.2.5 The Enhancement of Cytotoxicity of DMS by Inhibitors of ADPR-transferase

Inhibition of poly(ADP-ribose) biosynthesis, by a number of competitive inhibitors (Purnell & Whish, 1980), blocks the DNA damaging agent induced lowering of cellular NAD content. They also retard the excision repair of DNA and causes a synergistic enhancement of the cytotoxicity of DNA damaging agents (Skidmore et al., 1979; Nduka et al., 1980 and Durkacz et al., 1980).

Following the investigation of cytotoxicity of DMS in T.brucei; the enhancement of cytotoxicity of this DNA damaging agent by the competitive inhibitors of the enzyme ADPR-transferase, 3-aminobenzamide (3AB) and 3-acetamidobenzamide (3AAB) together with their non inhibitory acid analogues 3-aminobenzoic acid (3ABA) and 3-acetamidobenzoic acid (3AABA) respectively, was investigated.

Trypanosomes were pre-incubated with the inhibitors or their acid analogues at 2 mM with a density of 5×10^7 cells/ml of culture medium at 37°C for 30 minutes. DMS was added to a variety of final concentrations varying from 50 μ M to 1 mM. The resulting cultures were mixed well and incubated at 37°C in a water bath. Samples (1 ml) were removed and cellular NAD levels were measured using the method of Nisselbaum & Green (1969) (see methods 3.2.2.1).

The inhibition of the cellular NAD drop using 2 mM 3AAB, was also studied in trypanosomes permeabilised to NAD. Trypanosomes at a density of approximately 1×10^8 cells/ml were treated with 100 μ M DMS for 30 minutes at 37°C prior to permeabilisation. The total ADPR-transferase activity was measured either in the absence (●) or presence (★) of the 3AAB in permeabilised trypanosomes. The inhibitor was added before the substrate, [3 H]-NAD, (Fig. 4.13).

Analysing the cellular NAD content after 3 hours DMS treatment in the presence of either 2 mM 3AB or 3AAB, indicates a dramatic retardation of the cellular NAD loss (Figs. 4.9,4.10,4.11,4.12). After 50 μ M, 0.1 mM and 0.2 mM DMS treatment (Fig. 4.9,a,b,c and d) in the presence of 2 mM 3AB the cellular NAD losses were found to be 17%, 39% and 48% whereas without the inhibitor the losses are 52%, 80% and 86% respectively. In the presence of 2 mM 3AAB (Fig. 4.10,a,b,c and d), the cellular NAD losses after 3 hours DMS treatment; 50 μ M, 0.1mM and 0.2mM; were found to be 9%, 13% and 24% respectively. This shows that in the presence of ADPR-transferase inhibitors the cellular NAD drop is greatly retarded compared to the inhibitor free experiments (48%, 78% and 85% respectively) using the same DMS concentrations.

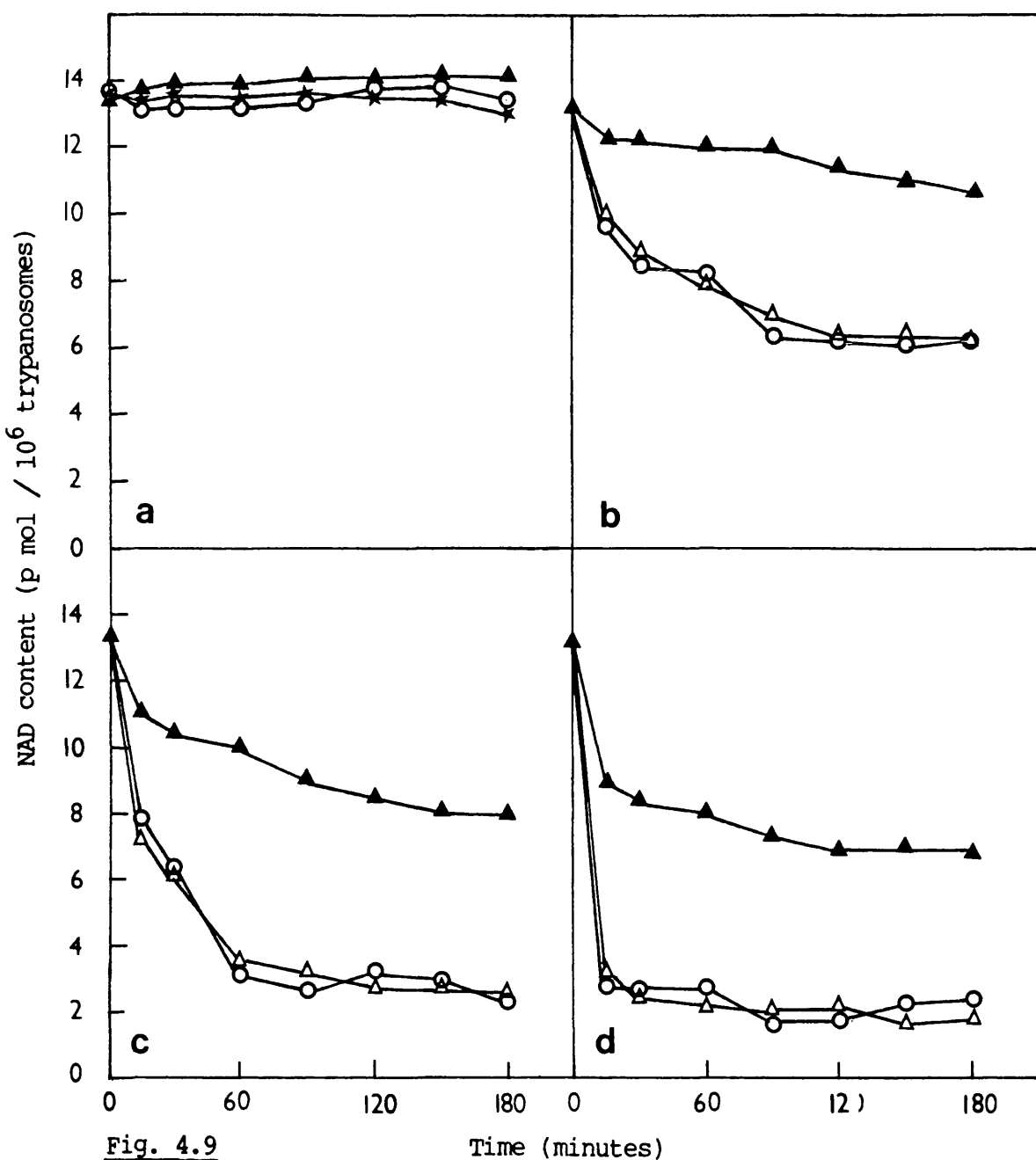


Fig. 4.9

Time (minutes)

Effect of various concentrations of DMS on the cellular NAD content in the absence or presence of 3AB or 3ABA. The NAD content in trypanosomes treated with no DMS(a,★), or 50 μ M (b), 0.1 mM (c) and 0.2 mM (d) DMS for 3 hr in the absence (Δ) or presence of 2 mM 3AB (\blacktriangle) or 3ABA (\circ) was estimated (in the 3AB and 3ABA treated samples, prior to the DMS addition, the trypanosomes were pre-incubated with 2 mM 3AB or 3ABA for 30 minutes at 37°C).

Number of determinations = 4.

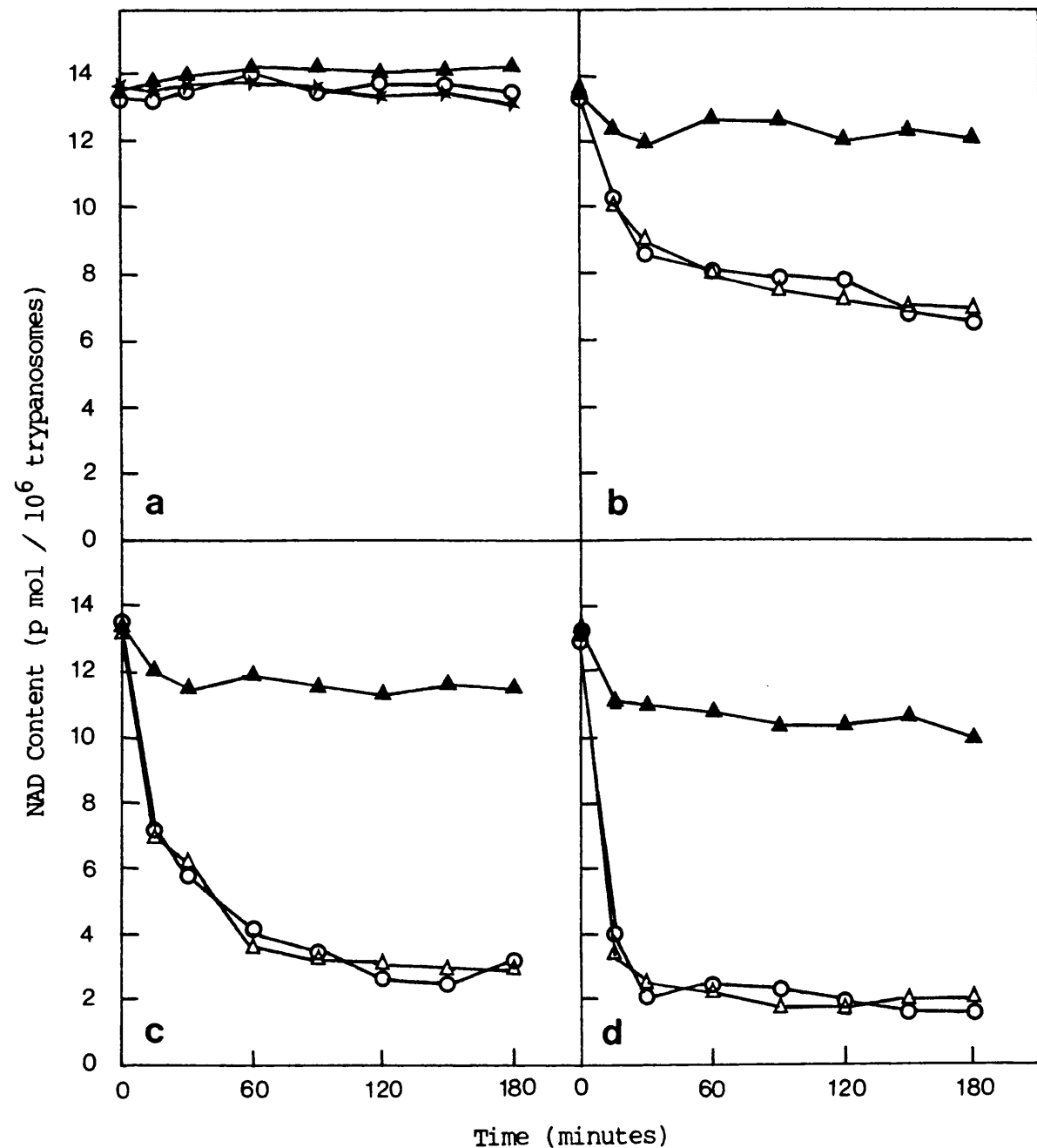


Fig. 4.10

Effect of various concentrations of DMS on the cellular NAD content in the absence or presence of 3AAB or 3AABA. The NAD content in trypanosomes treated with no DMS (a, ★), or 50 μM (b), 0.1 mM (c) and 0.2 mM (d) DMS for 3 hr in the absence (Δ) or presence of 2 mM 3AAB (▲) or 3AABA (○) was estimated (in the 3AAB and 3AABA treated samples, prior to the DMS addition, the trypanosomes were pre-incubated with 2 mM 3AABA for 30 minutes at 37°C).

Number of determinations = 4.

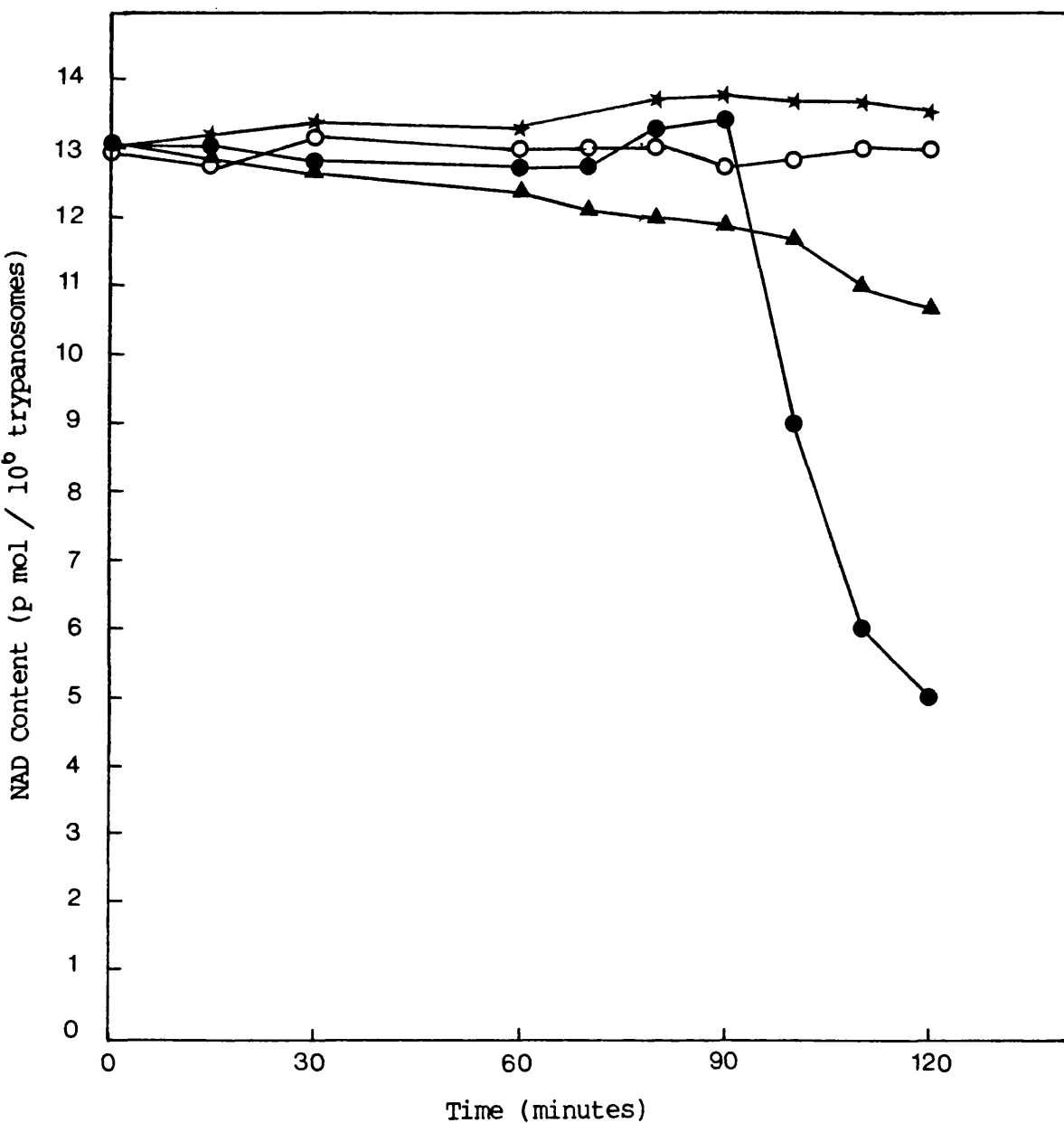


Fig. 4.11

Effect of 1 mM DMS on the cellular NAD content in T.brucei in the presence or absence of 3AAB. The NAD content in trypanosomes treated with 1 mM DMS either in the presence or absence of 2 mM 3AAB (prior to the DMS treatment the trypanosomes were pre-incubated with 2 mM 3AAB for 30 min. at 37°C) was estimated using the method of Nisselbaum & Green (1969). NAD level in control cells (O), 2 mM AAB (★), 1 mM DMS (●), 1 mM DMS+2 mM 3AAB (▲).

Number of determinations = 6.

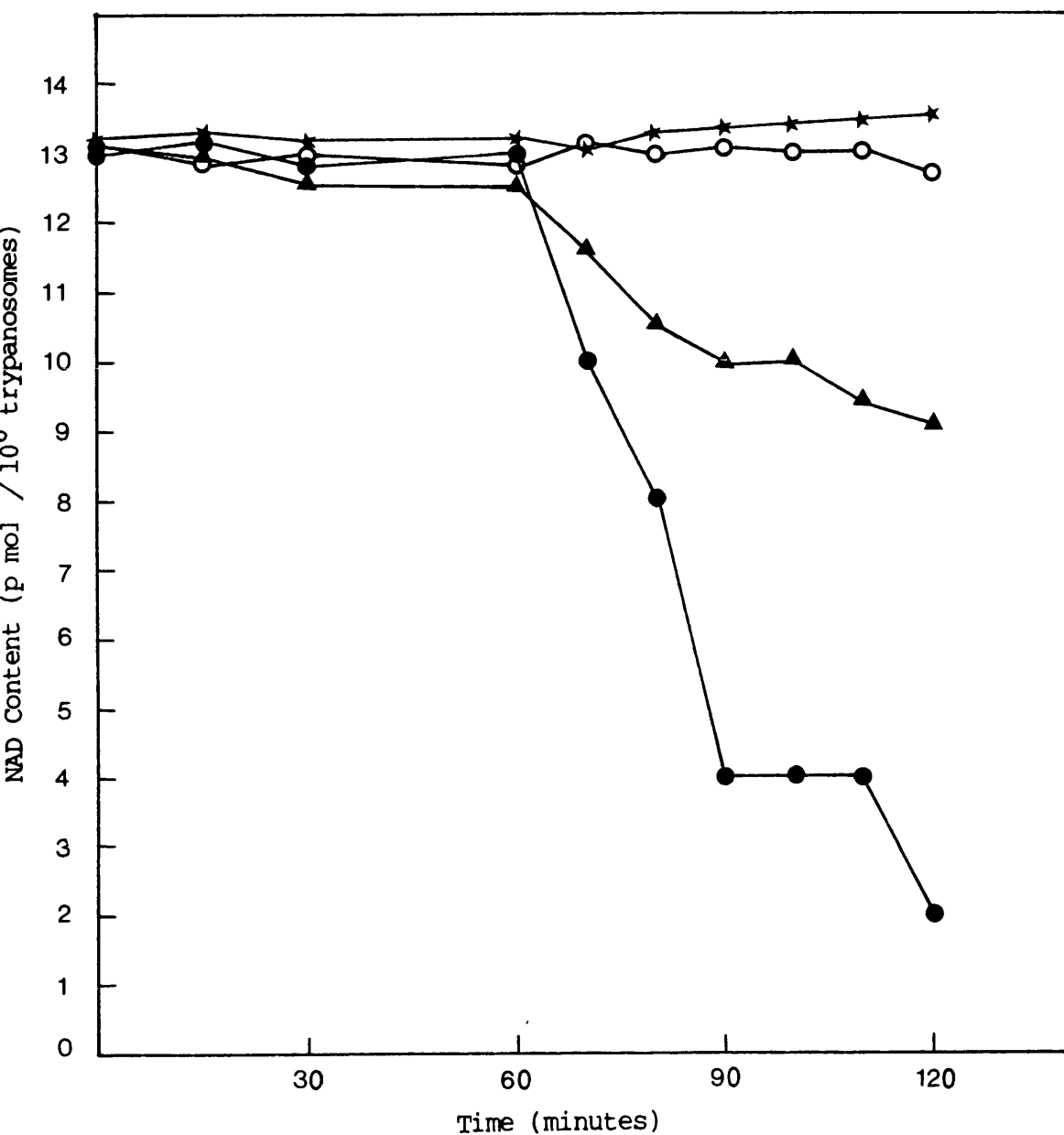


Fig. 4.12

Effect of 0.5 mM DMS on the cellular NAD content in T.brucei in the presence or absence of 3AAB. The NAD content was measured in trypanosomes which had been treated (or not) with 2 mM 3AAB (prior to the DMS treatment the trypanosomes were incubated with 2 mM 3AAB for 30 min. at 37°C) was estimated by the method of Nisselbaum & Green (1969). NAD level in control cells (O), 2 mM 3AAB (★), 0.5 mM DMS (●), 0.5 mM DMS+2 mM 3AAB (▲).

Number of determinations = 6

The NAD drop caused by DMS treatment at any of the concentrations used is blocked by the ADPR-transferase inhibitors 3AB or 3AAB. This was the case even though there is a considerable qualitative difference in the way that high or low concentrations of DMS affects the NAD levels in this organism (see Figs. 4.9, 4.10, 4.11, 4.12 and 4.13) In the absence of DMS treatment the inhibitors on their own did not cause a drop but instead a slight increase in the cellular NAD level.

In contrast to the inhibitors of ADPR-transferase activity, corresponding concentrations of the non-inhibitory acid analogues, 3ABA and 3AABA did not inhibit the DMS induced lowering of cellular NAD level (Fig. 4.9 & 4.10).

In the studies of the effect of ADPR-transferase inhibitors on the cellular NAD drop, 3AAB had the most dramatic effect which is compatible with the published K_i values of these inhibitors ($k_i=0.43 \mu\text{M}$ and $2.6 \mu\text{M}$ for 3AAB and 3AB respectively (Purnell & Whish 1980)).

ADPR-transferase assays (Fig. 4.13) also demonstrated that the initial rate of ADPR-transferase in trypanosomes treated with 0.1 mM DMS is 10-fold higher than the initial rate in trypanosomes whose ADPR-transferase activity was assayed in the presence of 2 mM 3AAB.

4.2.6 The Effect of γ -irradiation on the Cellular NAD Level in *T.brucei*

Induction of DNA damage in trypanosomes causes a drop in the cellular NAD level not only in response to the monofunctional alkylating agent DMS but also by exposure to γ -radiation. To study the effect of γ -irradiation on their cellular NAD level,

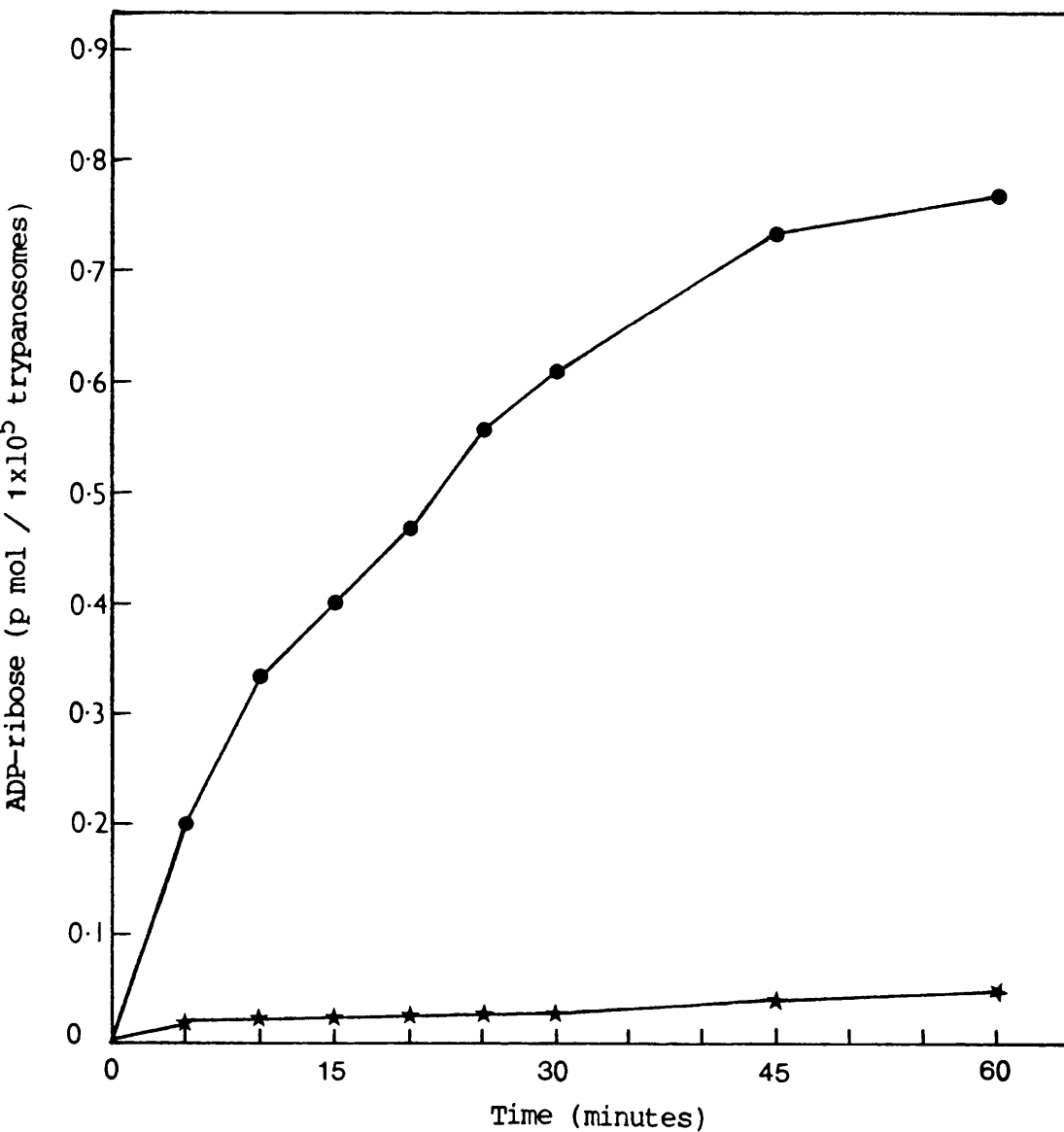


Fig. 4.13

Inhibition of ADPR-transferase activity in T.brucei permeabilised to NAD by hypotonic shock. Freshly isolated trypanosomes were made permeable to [^3H]-NAD (see methods). The potential ADPR-transferase activity was measured either in the absence (●), or presence of 2 mM 3AAB (★). The inhibitor was added to the permeabilised trypanosomes before the addition of [^3H]-NAD. Trypanosomes were treated with 0.1 mM DMS at 37°C for 30 minutes prior to permeabilisation. Each point is the mean of 4 measurements in 2 separate experiments.

trypanosomes at a density of 5×10^7 cells/ml were exposed to approximately 1.5, 2.5 and 15K rads of γ -radiation (see methods 3.2.2.4) (Fig. 4.14). In contrast to the DMS treatment, the radiation induced NAD drop is very rapid, usually occurring within 5 minutes of exposure to 1.5, 2.5 and 15K rads in a dose dependent manner. The NAD level reached approximately 73% , 54% and 43% of the control level, respectively (Fig. 4.14). Also consistent with the demonstration that γ -irradiation induced damage is rapidly repaired in many eukaryotic cells such as mouse L1210 cells (Skidmore et al., 1979), in trypanosomes this drop is also rapidly followed by restoration of NAD level to the control values usually within about 15-30 minutes even in parasites treated with 15K rads of γ -radiation. The decrease in the cellular NAD in mouse L1210 cells has been shown to be correlated with an enhanced specific activity of ADPR-transferase in these cells (Skidmore et al., 1979).

4.2.7 Enhancement of Cytotoxicity of γ -radiation by Inhibitors of ADPR-transferase

As in DMS induced reduction in the level of cellular NAD, the γ -induced drop in cellular NAD level is also partially blocked by the ADPR-transferase inhibitor 3AAB at 2 mM. This inhibition is observed at γ -radiation doses ranging from 1.5 to 15k rads. The NAD drop at all doses of γ -radiation used in this study, 1.5, 2.5 and 15k rads, in the presence of 2 mM 3AAB is 3-fold lower than the NAD level in comparable trypanosomes without inhibitor (Figs. 4.15, 4.16 and 4.17).

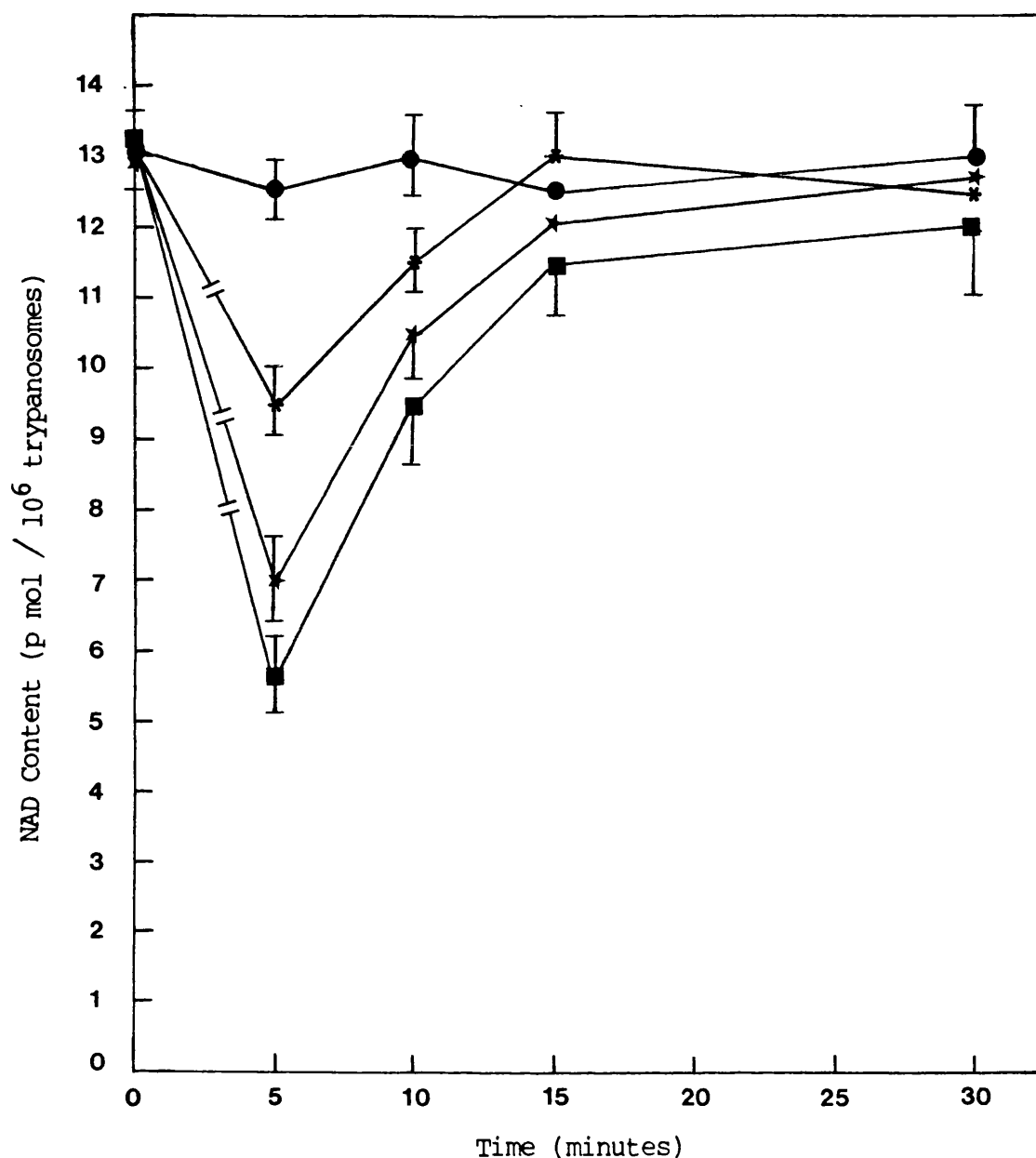


Fig. 4.14

The time course of the effect of various doses of γ -radiation on the cellular NAD content. The NAD content was measured at 37°C after exposure to various doses of γ -radiation; 1.5, 2.5 and 15k rads. γ -irradiation was carried out at room temperature. The resulting NAD levels were estimated by the method of Nisselbaum & Green (1969). The NAD level in control (un-irradiated) cells (●), 1.5k rads of γ -radiation (*), 2.5k rads of γ -radiation (★), 15k rads of γ -radiation(■). Each point is the mean of 6 measurements in 3 separate experiments, bars indicate the standard error of the mean.

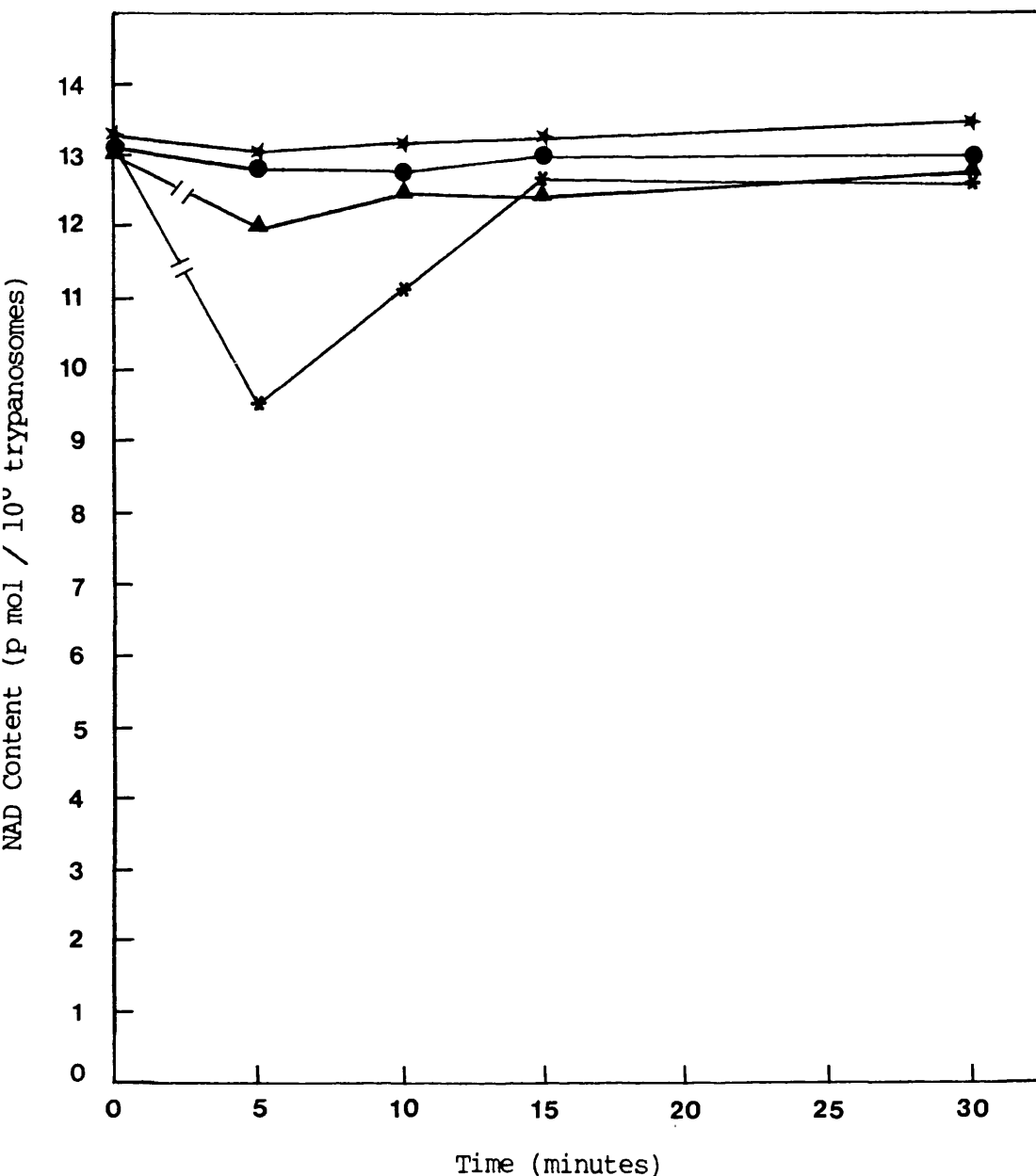


Fig 4.15

The time course of the effect of 1.5k rads of γ -radiation on the cellular NAD content in the presence of 3AAB. The NAD content in trypanosomes (5×10^7 cells/ml) exposed to 1.5k rads of γ -radiation (γ -irradiation was carried out at room temperature, see methods) either in the presence or absence of 3AAB (prior to the γ -irradiation the trypanosomes were incubated with 2 mM 3AAB for 30 min. at 37°C), was estimated by the method of Nisselbaum & Green (1969). NAD level in un-irradiated cells (●), 1.5k rads of γ -radiation (*), 2 mM 3AAB (★), 1.5k rads of γ -radiation+2 mM 3AAB (▲). Number of determinations = 6.

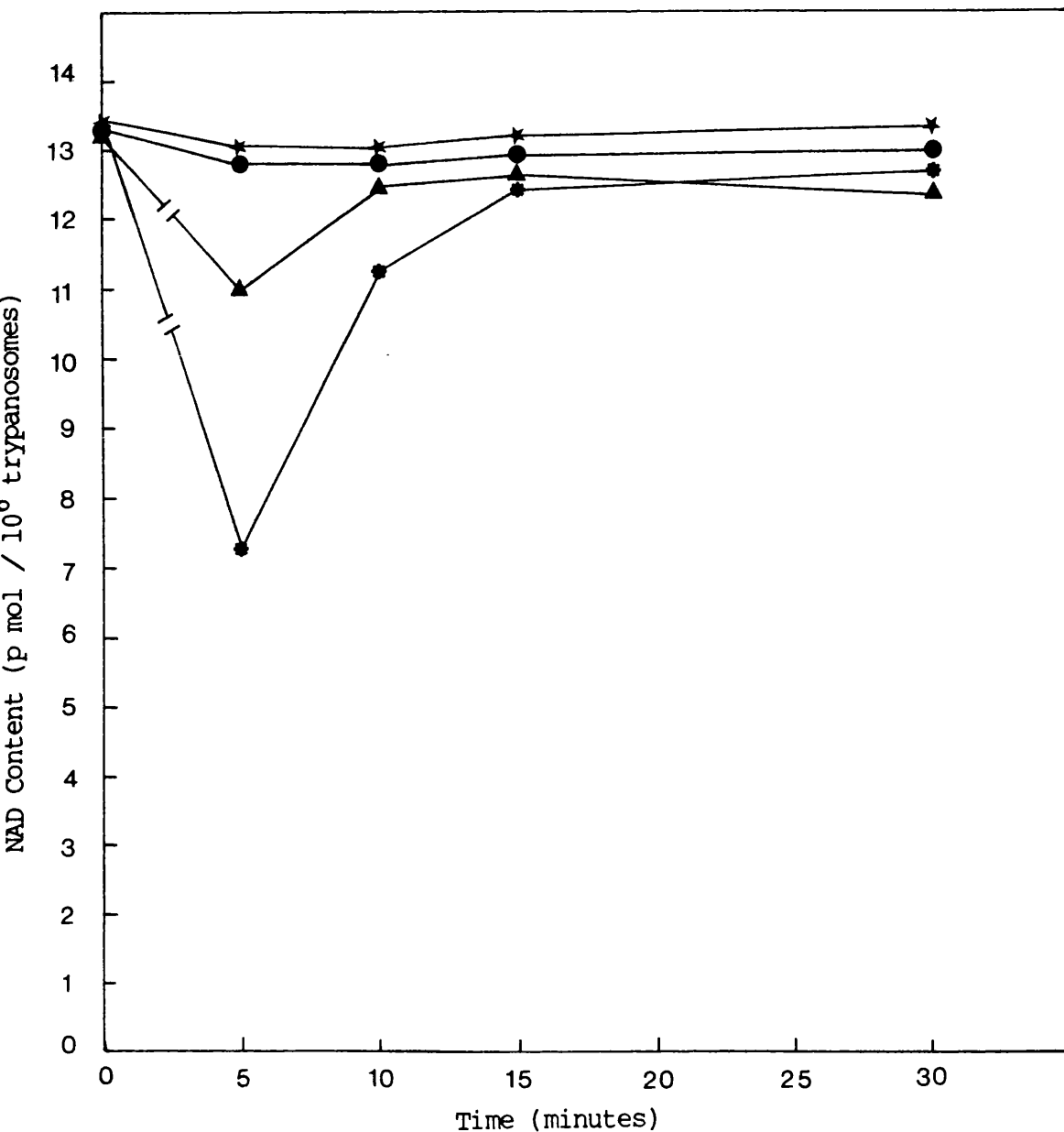


Fig. 4.16

The time course of the effect of 2.5k rads of γ -radiation on the cellular NAD content in the presence of 3AAB. The NAD content in trypanosomes (5×10^7 cells/ml) exposed to 2.5k rads of γ -radiation (γ -irradiation was carried out at room temperature, see methods) either in the presence or absence of 3AAB (prior to the γ -irradiation the trypanosomes were incubated with 2 mM 3AAB for 30 min. at 37°C) was estimated by the method of Nisselbaum & Green (1969). NAD level in un-irradiated cells (●), 2.5k rads of γ -radiation (●*), 2 mM 3AAB (★), 2.5k rads of γ -radiation+2 mM 3AAB (▲). Number of determinations = 6

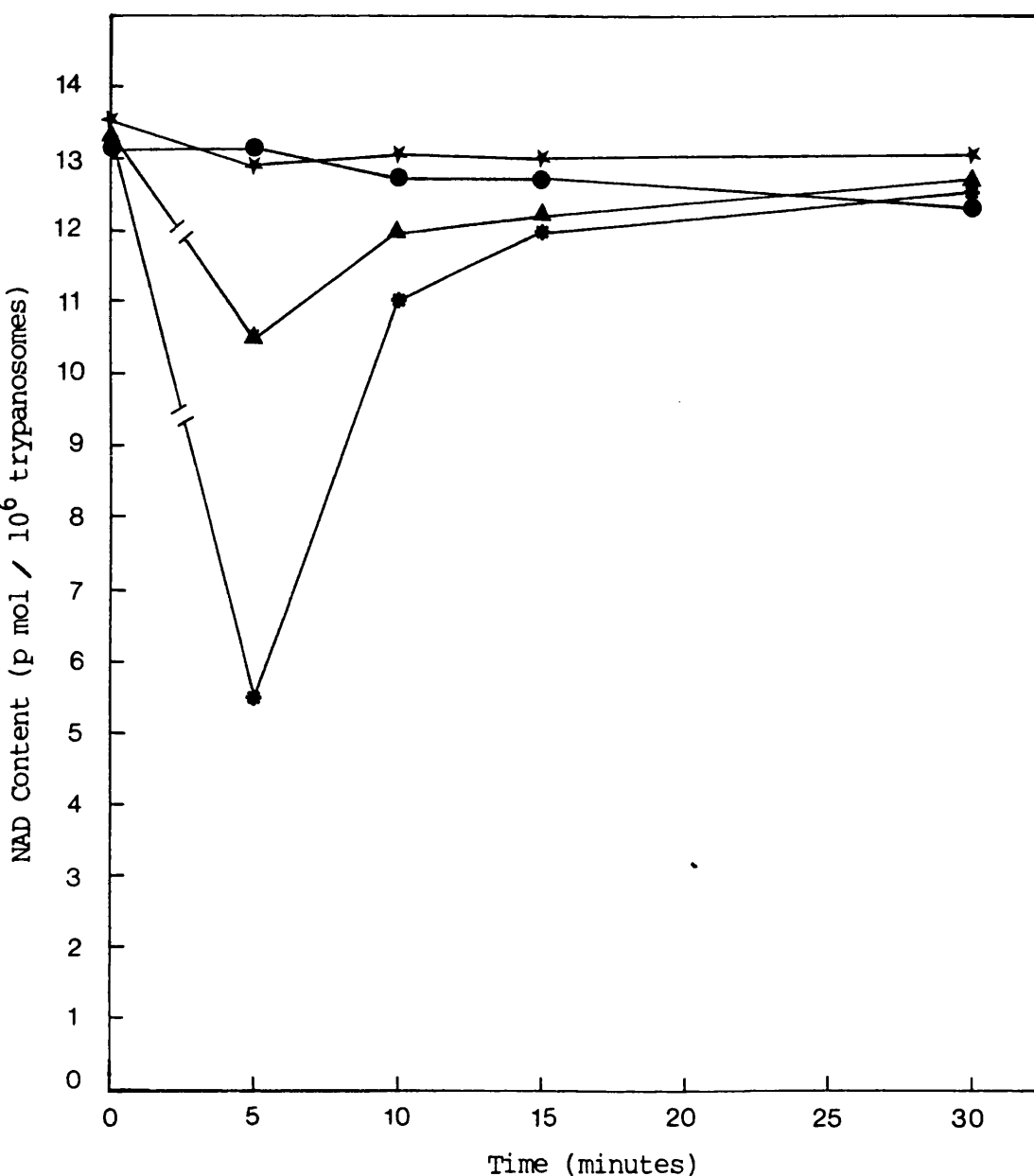


Fig. 4.17

The time course of the effect of 15K rads of γ -radiation on the cellular NAD content in the presence of 3AAB. The NAD content in trypanosomes (5×10^7 cells/ml) exposed to 15K rads of γ -radiation (γ -irradiation was carried out at room temperature, see methods) either in the presence or absence of 3AAB (prior to the γ -irradiation on the trypanosomes were incubated with 2mM 3AAB for 30 min. at 37°C), was estimated by the method of Nisselbaum & Green (1969). NAD level in un-irradiated cells (●), 15K rads of γ -radiation (●), 2mM 3AAB (★), 15K rads of γ -radiation+2 mM 3AAB (▲). Number of determinations = 6.

4.2.8 DNA Molecular Weight Studies

DNA damage and repair, following DMS treatment and γ - irradiation was investigated in T.brucei by measuring the sedimentation rate of trypanosomal nucleoids according to the method of Farzaneh et al. (1985). The principle of the method is a gentle lysis of the cells in the presence of non-ionic detergents and high salt concentrations on the top of gradient without subsequent manipulation. When cells are lysed in this way, structures resembling nuclei, called nucleoids form. The nucleoids contain essentially all of the nuclear DNA, most of the RNA but are grossly depleted in proteins. The DNA is in a supercoiled configuration and therefore compact; consequently the nucleoids sediment rapidly in a neutral sucrose gradient. The nucleoids consist apparently of large numbers of independent, supercoiled DNA loops. One single or double-strand nick in a DNA supercoiled loop will relieve the supercoiling and expand the loop, or break it into uncoiled strands if the break is double-stranded. Consequently, single-strand nicks generate a less compact structure which sediments more slowly in the neutral sucrose gradients. When the DNA is repaired the nick is resealed and the loop is supercoiled again. Thus, the nucleoid again becomes compact and sediments rapidly. To identify the position of nucleoid bands; gradients containing ethidium bromide, are visualised with an ultraviolet transilluminator. Ethidium bromide removes the negative superhelical turns from DNA, but at high (saturating) concentrations (30 $\mu\text{g/ml}$) it establishes positive ones, so the sedimentation behaviour of the DNA is similar to that at zero concentration of the dye.

This technique was used to assess the effect of inhibition of poly(ADP-ribose) biosynthesis by 3AAB on the capacity to repair DNA

of trypanosomes following damage by DMS and γ -radiation.

4.2.8.1 The Effect of DMS Treatment and γ -irradiation on the Sedimentation Rate of Nucleoids in T.brucei

Trypanosomes were maintained at 5×10^8 cells/ml in HEPES buffered RPMI 1640 medium supplemented with 10% (V/V) fetal calf serum. All experiments were started at a cell density of about 5×10^8 trypanosomes/ml. Trypanosomes treated with 2 mM 3AAB were incubated in its presence for 30 minutes at 37°C prior to the start of the experiments and the inhibitor was present throughout the study.

DMS was made up in 50% (V/V) ethanol/water (final ethanol concentration 0.25% (V/V)). To 1 ml aliquots of trypanosomes (5×10^8 cells/ml) DMS was added to a final concentration of 100 μ M and 50 μ M, as indicated. The resulting cultures were mixed well and incubated for 1 hour at 37°C in a water bath.

γ -irradiation was performed at room temperature in a cobalt-60 source (see methods 3.2.2.4). Trypanosomes were exposed to 15k rads of γ -radiation and were either immediately placed on ice or incubated at 37°C for the indicated times.

For studies of the effect of DMS treatment and γ -irradiation on the nucleoid sedimentation rate 100 μ l (5×10^7 trypanosomes/ml) of each culture was gently deposited in 300 μ l of lysis solution which had already been overlaid on 14 ml 15-30% linear sucrose gradients. The gradients were kept in the dark for 30 minutes at room temperature and then centrifuged at 23k rpm (approximately $100 \times 10^3 g$) in a Beckman SW 27 rotor for 2 hours at room temperature (20°C).

All centrifugations were performed in the presence of ethidium bromide (30 μ g/ml). Using the fluorescence of the EtBr-DNA complex

the position of the nucleoids is easily identified. All centrifugations included tubes with undamaged (reference) nucleoids. The migration ratio was estimated as described in methods (3.2.2.5).

The effect of damage induced by DMS and γ -radiation on sedimentation of nucleoids derived from T.brucei is shown in tables (4.2) and (4.3) respectively. The relative sedimentation rate decreased with increasing concentration of DMS from 50 μ M to 100 μ M; presumably due to the release of the supercoiling within the nucleoids, leading to a more relaxed and open structure.

The 1 hour treatment of trypanosomes at 37°C with either 50 μ M or 100 μ M DMS reduced the nucleoid sedimentation rate by half. In the presence of 3AAB the sedimentation rate of nucleoids from the DMS treated trypanosomes is even slower (table 4.2), indicating the presence of a larger number of breaks. Table (4.3) demonstrates that irradiation of trypanosomes with 15k rads of γ -radiation also reduced the relative sedimentation rate to 0.36. 30 minutes post-irradiation incubation of these cells, at 37°C, in the absence of ADPR-transferase inhibitors resulted in the partial restoration of the relative sedimentation rate to 0.8. However, in the presence of 3AAB the sedimentation rate of nucleoids increases from approximately 0.3 to 0.6. This relatively lower increase in sedimentation rate in the presence of 3AAB indicates a reduced rate of strand-break ligation in the presence of inhibitor.

Table 4.2 The effect of DMS treatment (50µM & 100µM)
on the nucleoid sedimentation rate of
T.brucei in the presence or absence of
2mM 3AAB relative to untreated cells.

Experiments	Relative Migration Ratio (with respect to untreated cells) 1 hr DMS Treatment at 37°C
Control	1
2mM 3AAB	0.98
50µM DMS	0.58
50µM DMS +2mM 3AAB	0.44
100µM DMS	0.55
100µM DMS +2mM 3AAB	0.44

Results are the means of two independent experiments.

Table 4.3 The effect of γ -irradiation (15K rads) on the nucleoid sedimentation rate of T.brucei in the presence or absence of 2mM 3AAB relative to untreated cells.

Experiments	Relative Migration Ratio (with respect to untreated cells)
Control	1
2mM 3AAB	0.96
15K rads γ -radiation immediate lysis (no post-incubation)	0.36
15K rads γ -radiation 30 min post-incubation at 37°C	0.8
15K rads γ -radiation +2mM 3AAB immediate lysis (no post-incubation)	0.3
15K rads γ -radiation +2mM 3AAB 30 mins post-incubation at 37°C	0.6

Results are the means of two independent experiments.

4.3 Discussion

Results presented in this section demonstrate that exposure of trypanosomes to DNA damaging agents, DMS and γ -radiation, increases the activity of ADPR-transferase in T.brucei. DMS, a mono-functional methylating agent lowered the cellular NAD content in a dose-dependent manner up to 300 μ M reaching the minimum level in 1 to 2 hours of treatment. However, higher concentrations of DMS, 0.5 mM and 1 mM, had no effect on the cellular NAD level until some 70 and 90 minutes of treatment, respectively. After this time the NAD level fell in a linear fashion. This lag phase is probably due to the inactivation of the ADPR-transferase enzyme itself by the high concentration of DMS. The possibility of such an inactivation was investigated by measuring ADPR-transferase activity in trypanosomes permeabilised to NAD. The results suggest that incorporation of NAD into acid insoluble materials after treatment with 0.5 mM DMS was only 2 fold higher than the basal level detected in untreated trypanosomes. At the higher concentration of 1 mM DMS there is no increase in ADPR-transferase activity above that detected in untreated trypanosomes. These results contrast with the 3.5 fold higher activity of the enzyme in trypanosomes treated with the lower concentration of 100 μ M. Interestingly, the enzyme activity was found to be maximal when the cellular NAD content was decreasing at the highest rate (Figs. 4.6 & 4.8). The delayed onset of NAD reduction in cells treated with high concentrations of DMS could of course be also due to the direct methylation of the ADPR acceptor proteins rather than the enzyme itself, thus reducing their efficiency or capacity as acceptors of ADP-ribosylation.

Several types of mammalian cells are able to rejoin the DNA double strand-breaks produced by ionizing radiation (Lehman 1978).

The γ -radiation used in this work also resulted in a dose-dependent drop in cellular NAD levels. The minimum cellular NAD level was reached in about 5 minutes, the earliest time tested after γ -irradiation. From this time onwards the NAD level rises reaching near normal values within 15 minutes of treatment. It has been observed that the rapid drop in mammalian cellular NAD level after γ -irradiation is associated with an even more rapid increase in the activity of the enzyme (Skidmore et al., 1979).

This correlation between the drop in cellular NAD levels and the activation of nuclear ADPR-transferase activity has been confirmed in several laboratories using a variety of DNA damaging agents.

Several inhibitors of nuclear ADPR-transferase prevented the cellular NAD drop induced by DNA damaging agents. Various benzamides substituted in the 3-position were the most inhibitory compounds found (Purnell & Whish, 1980). The benzamide analogues, 3-aminobenzamide, 3-methoxybenzamide and 3-acetamidobenzamide are competitive inhibitors of the enzyme. The possibility that 3-aminobenzamide and 3-methoxybenzamide work at the polymerisation step, but not at the initiation step, has been suggested (Surowy & Whish 1980). These inhibitors are relatively specific in that at non-toxic concentrations they do not appear to affect other biochemical pathways. They are therefore valuable probes for assessing the effect of inhibition of nuclear ADPR-transferase activity in vitro.

In contrast, non inhibitory chemical analogues of the above inhibitors do not prevent the drop in cellular NAD content (Skidmore et al., 1979; Durkacz et al., 1980 and Sims et al., 1982). Treatment of the cells with the enzyme inhibitors either alone or after DNA

damage resulted in a small increase in the NAD level above the normal levels. In this work the inhibitory effect of 2 mM 3AB and 3AAB on ADPR-transferase activity was examined. Both inhibitors gave basically the same result. But 3AAB seems to be a more potent inhibitor of ADPR-transferase activity. In the presence of 3AAB the cellular NAD drop was substantially blocked at all DMS concentrations used. There was a small rise in cellular NAD content when these inhibitors were used alone (Figs. 4.9,4.10,4.11 and 4.12). These results confirm that DNA damage activates ADPR-transferase activity and thereby lowers the cellular NAD level. It has been reported that the extent of the inhibition of the NAD drop is dependent on the concentration of the enzyme inhibitor and on the amount of damage (Skidmore et al., 1979). 2 mM 3AAB used in this investigation completely inhibited the cellular NAD drop caused by 1.5K rads of γ -radiation, however this concentration of 3AAB only partially inhibited the effect of higher doses of γ -radiation (2.5 and 15K rads).

Analysis of the effect of ADPR-transferase inhibitors on the nucleoid sedimentation rate of trypanosomes treated with DMS or γ -radiation demonstrates the involvements of ADPR-transferase activity in the repair of both types of DNA damage.

The 1 hour treatment of cells with either 50 μ M or 100 μ M DMS reduces the nucleoid sedimentation rate by half. In the presence of 3AAB the sedimentation rate of nucleoids from the DMS treated trypanosomes is even slower, indicating the presence of a larger number of breaks.

DMS has a short half-life (approximately 15 minutes) at neutral pH. Therefore, the latter half of the 1 hour incubation period could

be regarded as the post DMS treatment period. However, the formation of DNA strand-breaks after DMS treatment is the result of enzymatic removal of the damaged bases and the detected level of breaks at any one time is the net balance between the rate of formation and removal of breaks. Therefore although the reduced sedimentation rate after DMS treatment does indicate the presence of DNA strand-breaks, the further reduction in the sedimentation rate in 3AAB treated trypanosomes could be the result of either increased rate of strand-break formation or a decrease in the rate of strand-break ligation in the presence of the inhibitor. The present studies do not distinguish between the two possibilities. However, the weight of published evidence (see introduction) strongly suggests the reduced rate of strand-ligation as the likely effect of 3AAB treatment. This interpretation is further strengthened by the study of the sedimentation rates after γ -irradiation. Table (4.3) demonstrates that irradiation with 15k rads of γ -rays reduces the relative sedimentation rate to 0.36. 30 minutes post-irradiation incubation of these cells results in the partial restoration of the relative sedimentation rate to 0.8. Since the induction of DNA strand-breaks by γ -radiation, in contrast to DMS, is a physical event (independent of enzymatic formation of breaks) the maximum number of breaks are present immediately after irradiation. Therefore, the increase in the relative sedimentation rate during the post-incubation period does in fact indicate the repair and removal of DNA strand-breaks. The retardation of the increase in the sedimentation rate in the presence of 3AAB during the post-incubation period clearly demonstrates the involvement of ADPR-transferase in the repair of the γ -induced DNA strand-breaks in T.brucei.

It has been shown that, in mammalian cells, nuclear ADPR-transferase activity is stimulated by ionising radiation and a range of other agents, which bring about DNA strand breakage either directly or as a result of repair processes (For a review see Shall, 1984). However, only in some cases has the requirement of ADP-ribosylation for the subsequent repair of the DNA strand-breaks been directly demonstrated. For instance, Shall and his coworkers have reported that the ADPR-transferase inhibitor 3AB blocks the repair of DNA strand-breaks induced by the monofunctional alkylating agent DMS (Durkacz et al., 1980). They have also demonstrated that the regulation of DNA ligase activity by ADP-ribosylation of the enzyme may be the mechanism involved (Creissen & Shall, 1982). However the same group, and others, have reported that with ionising radiation ADPR-transferase inhibitors have only a small effect on DNA strand-break repair or cell survival (Nduka et al., 1980; James & Lehman, 1982 and Durkacz et al., 1981). There is some evidence that although the final extent of repair is unaffected the inhibitors of ADPR-transferase including 3AB may slow the resealing of X-ray induced DNA strand-breaks (Zwelling et al., 1982).

A role for ADP-ribosylation in the radiation response of cells was indicated by a report that ADPR-transferase activity is only weakly stimulated by radiation in Ataxia telangiectasia cells compared with the level of stimulation observed in cells from normal individuals which are much more radiation resistant (Edwards & Taylor, 1980). This observation suggested that inhibition of DNA synthesis in response to DNA damage was dependent on ADP-ribosylation. However, the involvement of ADP-ribosylation in the DNA synthesis inhibition response was not confirmed by James & Lehman (1982), who found that the ADPR-transferase inhibitor 3AB had little

effect on the inhibition of DNA synthesis by γ -irradiation.

In conclusion the studies carried out in T.brucei which are in agreement with the published data in other organisms and cells, demonstrate the activation of ADPR-transferase in cells treated with either DMS or γ -radiation. This increased activity results in a drop in cellular NAD (the specific enzyme substrate). The drop in cellular NAD level is blocked by the inhibition of the enzyme with the competitive inhibitors, 3AB and 3AAB. The inhibition of enzyme activity also blocks the repair of both DMS and γ -induced DNA strand-breaks. Based on the effect of the inhibitors on the repair of γ -induced breaks, the requirement for ADPR-transferase activity appears to be at the repair and ligation steps, rather than strand-break formation component of the repair processes.

Note added in preparation

Because these experiments were only carried out twice, the results need to be cautiously interperated.

CHAPTER FIVE

In vitro Cultivation of Trypanosoma brucei.brucei

Results and Discussion

5.1 Introduction

The development of in vitro culture systems has high priority in trypanosomiasis research. Progress achieved with African and South American trypanosomes opens new possibilities for various studies in vitro under controlled conditions.

The field of application is wide such as studies of metabolism and establishment of the nutritional requirements, screening of compounds to find new therapeutic drugs, production of antigens for serodiagnosis, study of differentiation processes which could be another method of control by blocking differentiation steps in the parasite's life cycle and replacement of the use of laboratory animals, or at least a reduction in their number.

Significant progress in the in vitro cultivation of African trypanosomes has been made during the last ten years. Most research activity has been concentrated on the species T.brucei containing the human pathogenic subspecies T.b.rhodesiense and T.b.gambiense. But work has also enabled the development of culture systems for the animal infective subspecies T.b.brucei, as well as T.congolense and T.vivax. Today most of the parasite stages in the life cycle of the above mentioned trypanosome species can be cultivated in vitro. However, with few exceptions, the culture systems are still inefficient in producing large numbers of parasites. That is, they rarely exceed a cell density greater than 10% of that found in the live animal with a fulminant infection.

In this study in vitro cultivation of the bloodstream forms of animal infective subspecies T.b.brucei here after referred to as T.brucei, (strain MITat 1.1) was attempted under 40 different culture conditions (2 different media, 5 different sera and in the presence or absence of 4 different feeder cells).

5.2 Results

5.2.1 In vitro Cultivation of Bloodstream Forms of T.brucei

The experiments were carried out in T-25 flasks with 10 ml of culture medium per flask. Incubation began with an initial concentration of 1×10^4 freshly isolated trypanosomes/ml of culture medium in the presence or absence of feeder cells. The cultures were incubated at 37°C in an atmosphere of 5% CO₂ and 95% air. In the absence of feeder cells trypanosomes become sluggish and did not increase in number. All organisms were dead after 48 hours of culture. In contrast, the trypanosomes in many flasks containing feeder cells increased in number and were still very motile after 24 hours of culture. The best culture medium which supported the long term growth of bloodstream forms of T.brucei was found to be DMEM supplemented with penicilin, streptomycin, glutamine, glucose (see materials 3.1.9b) and 10% (V/V) freshly prepared rabbit serum (see methods 3.2.1.2). The feeder cells which best supported the growth of these organisms were found to be Fisher rat embryonic fibroblasts (see materials 3.1.8d). For a summary of results see tables (5.1 and 5.2). The optimum age of the feeder cells was found to be between 24 and 48 hours (near confluent cultures). It seems that at least 24 hours was required by the feeder cells to overcome the effect of trypsinisation. It was also apparant that the Fisher rat embryonic fibroblast cells were most effective when just confluent. Commercially available rabbit sera did not support growth of bloodstream forms of T.brucei in these culture systems. The reason for this remains unknown.

When parasites were innoculated into just confluent Fisher rat embryonic fibroblast cultures, growth of the trypanosomes began

Table 5.1 Determination of optimal conditions for the growth of T.brucei bloodstream forms in medium RPMI 1640.

RPMI 1640 + sera	FEEDER CELLS															
	BHK 21				Rat spinal cord cells				Rat muscle fibroblasts				Fisher rat fibroblasts			
Hours	24	18	72	96	24	48	72	96	24	48	72	96	24	48	72	96
RPMI + H.S	1x10 ⁴	2x10 ⁴	0	0	1x10 ⁴	1x10 ⁴	0	0	1x10 ⁴	1x10 ⁴	1x10 ⁴	0	1x10 ⁴	1x10 ⁴	1x10 ⁴	0
RPMI + C.S	2x10 ⁴	2x10 ⁴	2x10 ⁴	2x10 ⁴	1x10 ⁴	2x10 ⁴	4x10 ⁴	3x10 ⁴	2x10 ⁴	3x10 ⁴	4x10 ⁴	4x10 ⁴	3x10 ⁴	5x10 ⁴	4x10 ⁴	4x10 ⁴
RPMI + F.C.S	6x10 ⁴	8x10 ⁴	5x10 ⁴	7x10 ⁴	4x10 ⁴	6x10 ⁴	5x10 ⁴	4x10 ⁴	7x10 ⁴	7x10 ⁴	7x10 ⁴	8x10 ⁴	4x10 ⁴	6x10 ⁴	7x10 ⁴	8x10 ⁴
RPMI + C.R.S	1x10 ⁴	0	0	0	1x10 ⁴	0	0	0	1x10 ⁴	0	0	0	1x10 ⁴	0	0	0
RPMI + F.R.S	6x10 ⁴	10x10 ⁴	10x10 ⁴	12x10 ⁴	5x10 ⁴	6x10 ⁴	10x10 ⁴	13x10 ⁴	4x10 ⁴	8x10 ⁴	10x10 ⁴	10x10 ⁴	6x10 ⁴	12x10 ⁴	12x10 ⁴	14x10 ⁴

H.S = Horse Serum C.S = Calf Serum F.C.S = Fetal Calf Serum

F.R.S = Freshly Prepared Rabbit Serum C.R.S = Commercial Rabbit Serum

Table 5.1

Cultures were initiated in T-25 flasks. 2-4 days old near-confluent feeder cell cultures were used. Each culture was inoculated with 1×10^4 freshly isolated trypanosomes/ml of RPMI 1640 medium. This culture medium was supplemented with 10% (V/V) of one of several different sera: horse, calf, fetal calf, commercial rabbit and freshly prepared rabbit serum together with the components outlined in the materials. The cultures were incubated in a humidified incubator at 37°C in 5% CO₂ and 95% air and the trypanosome density was estimated daily by using phase contrast microscopy, and active mobile trypanosomes were counted using a haemocytometer.

Table 5.1 Determination of optimal conditions for the growth of T.brucei bloodstream forms in medium RPMI 1640.

RPMI 1640 + sera	FEEDER CELLS															
	BHK 21				Rat spinal cord cells				Rat muscle fibroblasts				Fisher rat fibroblasts			
Hours	24	18	72	96	24	48	72	96	24	48	72	96	24	48	72	96
RPMI + H.S	1x10 ⁴	2x10 ⁴	0	0	1x10 ⁴	1x10 ⁴	0	0	1x10 ⁴	1x10 ⁴	1x10 ⁴	0	1x10 ⁴	1x10 ⁴	1x10 ⁴	0
RPMI + C.S	2x10 ⁴	2x10 ⁴	2x10 ⁴	2x10 ⁴	1x10 ⁴	2x10 ⁴	4x10 ⁴	3x10 ⁴	2x10 ⁴	3x10 ⁴	4x10 ⁴	4x10 ⁴	3x10 ⁴	5x10 ⁴	4x10 ⁴	4x10 ⁴
RPMI + F.C.S	6x10 ⁴	8x10 ⁴	5x10 ⁴	7x10 ⁴	4x10 ⁴	6x10 ⁴	5x10 ⁴	4x10 ⁴	7x10 ⁴	7x10 ⁴	7x10 ⁴	8x10 ⁴	4x10 ⁴	6x10 ⁴	7x10 ⁴	8x10 ⁴
RPMI + C.R.S	1x10 ⁴	0	0	0	1x10 ⁴	0	0	0	1x10 ⁴	0	0	0	1x10 ⁴	0	0	0
RPMI + F.R.S	6x10 ⁴	10x10 ⁴	10x10 ⁴	12x10 ⁴	5x10 ⁴	6x10 ⁴	10x10 ⁴	13x10 ⁴	4x10 ⁴	8x10 ⁴	10x10 ⁴	10x10 ⁴	6x10 ⁴	12x10 ⁴	12x10 ⁴	14x10 ⁴

H.S = Horse Serum C.S = Calf Serum

F.C.S = Fetal Calf Serum

F.R.S = Freshly Prepared Rabbit Serum

C.R.S = Commercial Rabbit Serum

Table 5.1

Cultures were initiated in T-25 flasks. 2-4 days old near-confluent feeder cell cultures were used. Each culture was inoculated with 1×10^4 freshly isolated trypanosomes/ml of RPMI 1640 medium. This culture medium was supplemented with 10% (V/V) of one of several different sera: horse, calf, fetal calf, commercial rabbit and freshly prepared rabbit serum together with the components outlined in the materials. The cultures were incubated in a humidified incubator at 37°C in 5% CO₂ and 95% air and the trypanosome density was estimated daily by using phase contrast microscopy, and active mobile trypanosomes were counted using a haemocytometer.

Table 5.2

Cultures were initiated in T-25 flasks. 2-4 days old near-confluent feeder cell cultures were used. Each culture was inoculated with 1×10^4 freshly isolated trypanosomes/ml of DMEM medium. This culture medium was supplemented with 10% (V/V) of one of several different sera: horse, calf, fetal calf, commercial rabbit and freshly prepared rabbit serum together with the components outlined in the materials. The cultures were incubated in a humidified incubator at 37°C in 5% CO₂ and 95% air and the trypanosome density was estimated daily by using phase contrast microscopy, and active mobile trypanosomes were counted using a haemocytometer.

Table 5.2 Determination of optimal conditions for the growth of T.brucei bloodstream forms in medium DMEM.

DMEM + sera	FEEDER CELLS															
	HK 21				Rat spinal cord cells				Rat muscle fibroblasts				Fisher rat fibroblasts			
Hours	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
DMEM + H.S	2×10^4	4×10^4	6×10^4	1×10^5	2×10^4	5×10^4	5×10^4	8×10^4	4×10^4	6×10^4	8×10^4	6×10^4	4×10^4	9×10^4	8×10^4	1×10^5
DMEM + C.S	2×10^4	6×10^4	1×10^5	1.5×10^5	2×10^4	7×10^4	1×10^5	1.2×10^5	3×10^4	8×10^4	1×10^5	1×10^5	4×10^4	1×10^5	1.2×10^5	1.5×10^5
DMEM + F.C.S	6×10^4	2×10^5	4.2×10^5	7.5×10^5	7×10^4	3.3×10^5	5.1×10^5	6.8×10^5	8×10^4	3.3×10^5	5.5×10^5	7.8×10^5	8×10^4	4×10^5	6.5×10^5	9.5×10^5
DMEM + C.R.S	1×10^4	0	0	0	1×10^4	0	0	0	2×10^4	0	0	0	1×10^4	0	0	0
DMEM + F.R.S	8×10^4	3.6×10^5	5.8×10^4	9.2×10^5	6×10^4	3.2×10^5	7.1×10^5	1.1×10^6	7×10^4	2.9×10^5	6.5×10^5	8.4×10^5	8×10^4	4.5×10^5	6.8×10^5	1.4×10^6

H.S = Horse Serum C.S = Calf Serum

F.C.S = Fetal Calf Serum

F.R.S = Freshly Prepared Rabbit Serum

C.R.S = Commercial Rabbit Serum

Table 5.2 Determination of optimal conditions for the growth of T.brucei bloodstream forms in medium DMEM.

DMEM + sera	FEEDER CELLS															
	HK 21				Rat spinal cord cells				Rat muscle fibroblasts				Fisher rat fibroblasts			
Hours	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
DMEM + H.S	2×10^4	4×10^4	6×10^4	1×10^5	2×10^4	5×10^4	5×10^4	8×10^4	4×10^4	6×10^4	8×10^4	6×10^4	4×10^4	9×10^4	8×10^4	1×10^5
DMEM + C.S	2×10^4	6×10^4	1×10^5	1.5×10^5	2×10^4	7×10^4	1×10^5	1.2×10^5	3×10^4	8×10^4	1×10^5	1×10^5	4×10^4	1×10^5	1.2×10^5	1.5×10^5
DMEM + F.C.S	6×10^4	2×10^5	4.2×10^5	7.5×10^5	7×10^4	3.3×10^5	5.1×10^5	6.8×10^5	8×10^4	3.3×10^5	5.5×10^5	7.8×10^5	8×10^4	4×10^5	6.5×10^5	9.5×10^5
DMEM + C.R.S	1×10^4	0	0	0	1×10^4	0	0	0	2×10^4	0	0	0	1×10^4	0	0	0
DMEM + F.R.S	8×10^4	3.6×10^5	5.8×10^4	9.2×10^5	6×10^4	3.2×10^5	7.1×10^5	1.1×10^6	7×10^4	2.9×10^5	6.5×10^5	8.4×10^5	8×10^4	4.5×10^5	6.8×10^5	1.4×10^6

H.S = Horse Serum C.S = Calf Serum

F.C.S = Fetal Calf Serum

F.R.S = Freshly Prepared Rabbit Serum

C.R.S = Commercial Rabbit Serum

rapidly. In such cultures, the trypanosomes multiplied exponentially with a doubling time of 6-7 hours. This doubling time is similar to that seen in vivo. The parasites multiplied to a density of approximately $1-2 \times 10^6$ trypanosomes/ml of culture medium (Fig. 5.1). This maximum cell yield is comparable to other culture systems with a number of other established cell lines (Brun et al., 1981a and 1984). The parasite numbers decreased exponentially after this density was reached (Fig. 5.1). In such cultures numerous dividing forms could be maintained for more than 3 days without changing the medium. In these cultures, the trypanosomes grew as typical long slender forms. Most of them were free in the culture supernatant while a few were associated with the feeder layer. In cultures where parasite numbers were diminishing following a period of maximum growth, the morphology of the trypanosomes was markedly altered; in particular they became very much shortened. Attempts were made to subculture these cultured trypanosomes at different stages of the growth period. Successful passages were usually obtained at 48 hour intervals from the previous cultures which contained predominantly long slender forms ($2-3 \times 10^5$ trypanosomes/ml of culture medium). The subcultivations were made by inoculation of fresh, nearly confluent feeder cells with 1×10^4 trypanosomes/ml of culture medium. Organisms subcultured in this way generally multiplied 2-4 fold during 24 hours. In such cultures, most of the organisms were very motile long slender forms. Numerous dividing forms were also present. Organisms in subcultures prepared from the inoculum in which long slender forms were not detected (late log phase) gradually died out, indicating that this shortened form of long slender trypanosomes was irreversibly senescent under this culture condition. Subcultivation was successfully carried on for 3 months, representing more than 20 passages and at least 150

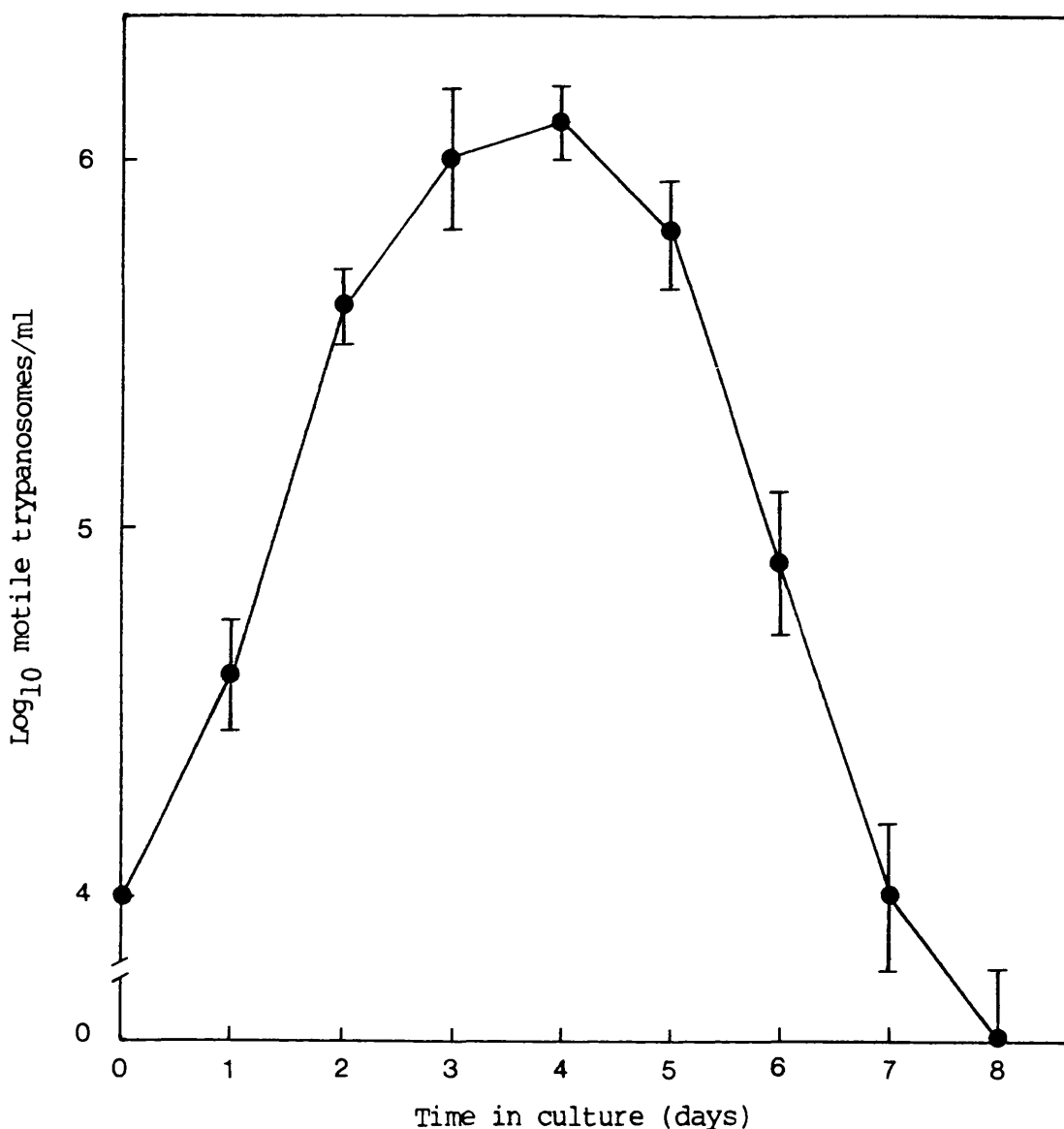


Fig. 5.1

Growth curve of T.brucei at 37°C. Growth of T.brucei (Strain MITat 1.1) bloodstream forms at 37°C in T-25 flasks with feeder cells (Fisher rat embryonic fibroblasts) in 10 ml DMEM together with the components outlined in materials and supplemented with 10% (V/V) freshly prepared and heat inactivated rabbit serum. The cultures were initiated with 1×10^4 freshly isolated bloodstream forms. Trypanosome motility was monitored daily using phase contrast microscopy. Each point is the mean of at least 6 independent experiments in each of which trypanosomes were counted in duplicates, the bars indicate the standard error of the mean.

population doublings. Figs. (5.2, a,b,c and d) show typical growth patterns of one such culture. These growth patterns varied considerably at 4 different passages depending on the predominant forms of the trypanosomes, the higher the fraction of the slender form trypanosomes the better was their survival and growth in culture. In (3 to 4 days) cultures there was an increasing fraction of the stumpy-looking trypanosomes which did not grow well when subsequently subcultured. In the early logarithmically growing cultures the trypanosomes were morphologically identical to those of the long slender forms of T.brucei in the bloodstream in vivo and retained their infectivity for rats when tested after 20,40,60 and 90 days of culture.

Baltz et al. (1985) reported the development of a semi-defined medium for the cultivation of bloodstream forms of T.brucei without using a feeder layer. Inclusion of 0.25 mM mercaptoethanol was reported to be crucial. Attempts to employ this in vitro culture system to study growth and differentiation of bloodstream forms of T.brucei without the interference of feeder cells failed although the experimental procedure was followed exactly as reported. Since the publication of the report by Baltz et al. in 1985 there has been no further report of the in vitro cultivation of T.brucei in the absence of feeder cells. No other group has yet reported the use of this system for the in vitro cultivation of T.brucei in the absence of feeder cells.

All four different feeder cells in combination with DMEM medium supplemented with 10% (V/V) freshly prepared rabbit serum allowed the propagation of trypanosomes. The reason that Fisher rat embryonic fibroblasts were preferred was that BHK-21 cells would not remain

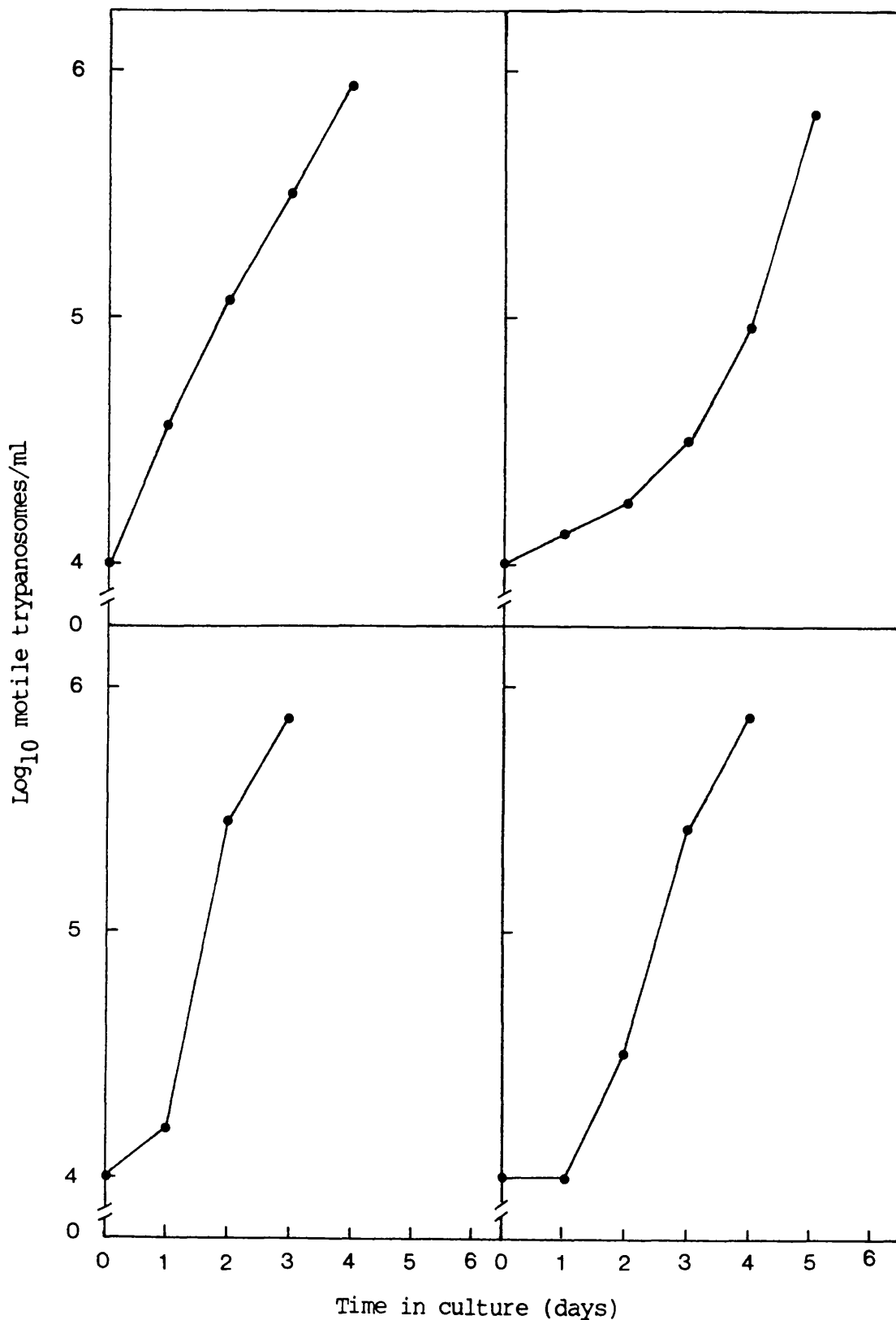


Fig. 5.2

Growth of subcultivated bloodstream forms of *T. brucei*. Subcultivations were carried out usually at 48 hours intervals from the previous cultures which contained predominantly long slender forms ($2-3 \times 10^5$ trypanosomes/ml of culture medium). The subcultivations were made by inoculation of fresh, nearly confluent feeder cells with 1×10^4 trypanosomes/ml of culture medium in T-25 flasks.

attached to T-25 flask for more than 3-4 days, by this time, these cells came off and the long term study of in vitro growth and survival of trypanosomes was not achieved. Rat spinal cord cells and rat muscle fibroblasts were not found to be suitable due to time consuming preparation and the long culture time required for obtaining confluent cultures of these cells (about 2 weeks). Fisher rat embryonic fibroblasts were easy to maintain and would reach confluence within 48 hours after being subcultured. Mammalian feeder layer cells have proven to be essential for the continuous cultivation of bloodstream forms of T.brucei (Hill et al., 1978a,b; Hirumi et al., 1977a and Tanner, 1980). A close trypanosome/feeder cells association is also required; this was indicated by the lack of growth stimulation by conditioned medium in the absence of feeder cells. This finding supports the investigation of Brun et al., 1981a and also Tanner, 1980, who studied extensively the mechanism of growth support of bloodstream forms of T.brucei by bovine fibroblast-like cells. Tanner found that a short-range interaction between trypanosomes and fibroblasts is obligatory and that growth of the bloodstream forms ceased whenever the trypanosomes were separated from the feeder layer cells. At present, the mechanism by which the feeder cells support the in vitro growth of bloodstream T.brucei is unknown.

5.3 Discussion

The culture conditions described here, DMEM supplemented with penicillin (200.I.U./l), streptomycin (200 µg/l) glutamine (10 mM), glucose (1% W/V) and 10% (V/V) freshly prepared rabbit serum (see methods 3.2.1.2) in the presence of Fisher rat embryonic fibroblasts (see materials 3.1.8d) has enabled the continuous cultivation of T.brucei (strain MITat 1.1). Using this culture system T.brucei could be maintained in culture continuously (at least three months). This represents approximately 150 population doublings, without any apparent loss of viability. These trypanosomes retained their infectivity for rats when tested after 20,40,60 and 90 days of culture.

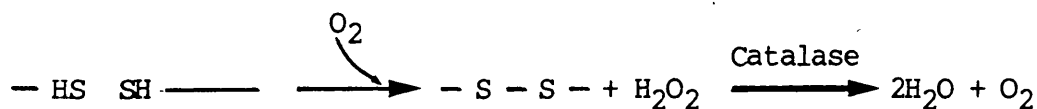
When growing trypanosomes in vitro, special attention has to be paid to retaining the parasite's natural characteristics. By selecting feeder cells and serum supplement from a susceptible host, a "natural" culture system can be obtained. In such a culture trypanosomes are more likely to grow and react in a comparable way to that in the vertebrate host. This is especially important for in vitro studies of metabolism and the effect of drugs. The results reported here demonstrate that under appropriate conditions the cultured trypanosomes maintained their bloodstream characteristics. Several conditions were tested and among these three parameters proved to be of a major importance. This included the presence of a feeder layer, the type of culture medium and the type of serum used. The co-cultivation of trypanosomes and mammalian cells was found to be essential for the in vitro growth and maintenance of the bloodstream forms. Optimal growth of the bloodstream form of T.brucei used in this study (MITat 1.1) was obtained in medium

supplemented with freshly prepared rabbit serum which also gave the best results with all the feeder layers. The need to use freshly prepared, as opposed to the commercially available, rabbit serum has also been suggested by Brun et al. (1981).

Due to the trypanosome's ability to adapt to environmental (culture) conditions, during the in vitro culture parasites may be produced which possess a divergent metabolism as compared to the parasites which grow in their natural hosts. Long-term maintenance of trypanosomes in culture may also lead to altered parasite populations which can no longer be considered to represent the parasites as they occur in the field. The constant selection in culture can easily change characteristics of the parasite population. Therefore it may prove important for most studies to use recently established cultures.

Note added in proof

Recently a new modified in vitro system for the culture of trypanosomes in the absence of feeder cells has been reported (Voorheis, H.P, Personal communication). This system also relies on addition of reducing agents such as mercaptoethanol, to a suitable culture medium. However it was found that the addition of these agents induce the production of a toxic level of hydrogen peroxide probably through the following chemical reaction:



To circumvent this toxic effect the addition of catalase was found to be necessary.

Suggestion for further work

It is important to note that the very late stages of the log growth of bloodstream forms of T.brucei in culture (at about $1-2 \times 10^6/\text{ml}$) appear to have a short-stumpy morphology. The sub-culturing of these stumpy looking trypanosomes into fresh cultures does not result in the resumption of growth. They therefore appear to be non-dividing. This rises the possibility that in fact they may represent the short-stumpy stage of transition from the bloodstream to procyclic differentiation. This possibility could be tested by the transfer of the late log phase trypanosomes to the differentiation culture (SDM-79, plus and minus cis-aconitate at 27°C) to determine if they would generate cultures of procyclic trypanosomes. If this were found to be true, it would enable the continuous culture of bloodstream trypanosomes and their growth and differentiation, through all the stages identified in the in vivo differentiation, into the procyclic forms. In addition, this would provide the possibility of in vitro study of the short-stumpy forms. This type of investigation has hitherto been hindered by the difficulty in obtaining a large number of trypanosomes in this stage of the life cycle from the infected animals.

CHAPTER SIX

The Enhancement of Cytotoxicity of DNA Damaging Agents by ADPR-transferase Inhibitors

(In vitro)

Results and Discussion

6.1 Introduction

Competitive inhibitors of ADPR-transferase, and in particular benzamides, have been used extensively in recent years as probes to elucidate the function of poly(ADP-ribose) in the cell. The results obtained have depended on the nature of the DNA damage involved and the test cell system used. Shall and his co-workers have investigated the effect of ADPR-transferase inhibitors on the response of L1210 mouse leukaemic lymphoblasts to alkylating agents and ionising radiation. For alkylating agents such as dimethyl sulphate and N-methylnitrosourea, treatment with ADPR-transferase inhibitors such as 5-methylnicotinamide and 3-aminobenzamide (3AB) was shown to enhance cell killing and inhibit DNA strand-break repair, but to have little or no effect on the killing effect of ionising radiation (Durkacz et al., 1980; Nduka et al., 1980 and Durkacz et al., 1981a). These observations were subsequently confirmed by James and Lehman (1982) using cultured human skin fibroblasts. Creissen and Shall (1982) suggested that inhibition of DNA strand-break repair induced by inhibitors of ADPR-transferase is due to the requirement of ADP-ribosylation for the stimulation of DNA ligase activity associated with repair. However, in contrast to these reports (lack of effect with ionising radiation) Zwelling et al. (1982) have found that 5-methylnicotinamide and 3AB slow the resealing of X-ray induced DNA strand-breaks in L1210 cells.

However studies of the cytotoxicity of ADPR-transferase inhibitors have mainly been concentrated on the effect of the continuous presence of inhibitors in agar (Durrant and Boyle, 1982 and Nduka et al., 1980). The survival assay technique was first developed by Puck et al., in 1956. Later Elkind and Witmore (1967) demonstrated that the proportion of cells surviving following

treatment with ionising radiation reflects the distribution of the lesions which prevent single cells from undergoing a sufficient number of divisions. Cells incapable of repetitive reproduction do not form macroscopic colonies and are thus not detected. Plating efficiency is used in this case to measure the effect of ADPR-transferase inhibitors and their acid analogues to enhance the cytotoxicity of various DNA damaging agents. In this study the effect of ADPR-transferase inhibitors 3AB and 3AAB, the effect of DNA damaging agents, DMS and γ -radiation, and also the cytotoxicity of the inhibitors alone and in combination with the DNA damaging agents on cultured T.brucei was investigated.

6.2 Results

6.2.1 The Effect of ADPR-transferase Inhibitors on the Survival and Growth of Cultured T.brucei

The effect of continuous presence of the specific inhibitors of ADPR-transferase on in vitro growth and survival of T.brucei was investigated. The competitive inhibitors of ADPR-transferase activity, 3AB and 3AAB were used. The experiments were carried out in T-25 flasks in the presence of feeder cells, Fisher rat embryonic fibroblasts (see materials 3.1.8d). The culture medium was DMEM together with the component outlined in materials (see materials 3.1.9b). This medium was supplemented with 10% (V/V) freshly prepared and heat inactivated rabbit serum (see methods 3.2.1.2). Incubation began with an initial cell density of 1×10^4 freshly isolated trypanosomes/ml of culture medium (10 ml in each flask). 3AB and 3AAB were added to the cultures, to obtain the indicated concentrations, at the time of incubation. The cultures were incubated at 37°C in an atmosphere of 5% CO₂ and 95% air.

In the continuous presence of 3AB there is little detectable retardation of growth up to concentrations as high as 5 mM, but at 10 mM there is clear evidence of cytotoxicity and/or inhibition of growth (Figs. 6.1 and 6.2). In the continuous presence of 3AAB up to 200 µM there is no detectable effect on the growth of trypanosomes. However at 500 µM 3AAB there is a small but clear inhibition of growth. The cytotoxic effect and/or inhibition of growth is very much more pronounced at higher inhibitor concentrations (Fig. 6.3).

The aim of this study was to find the highest inhibitor concentrations which were not cytotoxic to cultured trypanosomes in

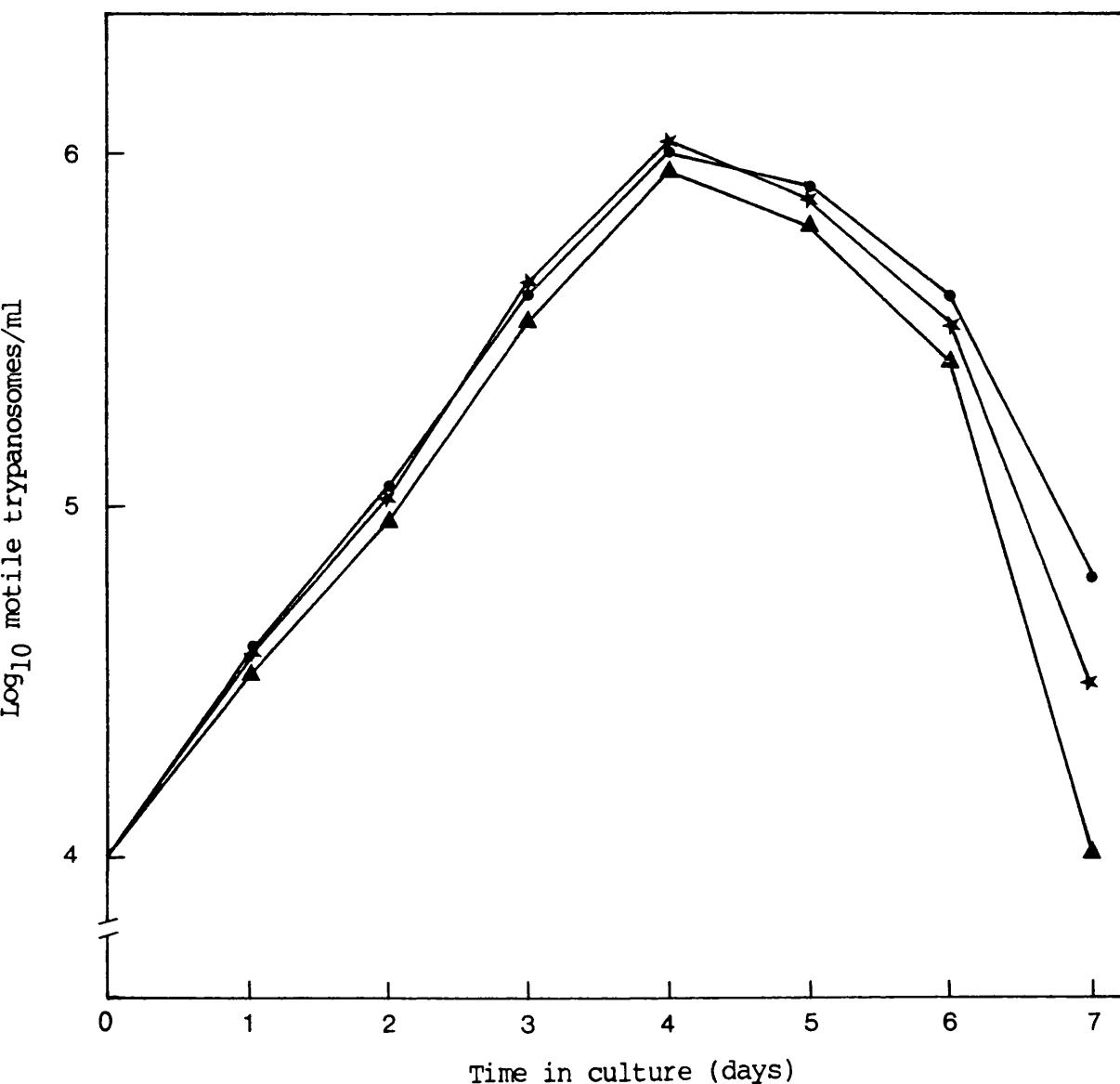


Fig. 6.1

The effect of 3AB on cultured T.brucei. Trypanosomes were cultured at an initial cell density of 1×10^4 trypanosomes/ml. 3AB was added to the cultures at the time of incubation. The number of viable trypanosomes were estimated by counting the motile organisms using a haemocytometer and phase contrast microscopy. Control cells (●), 500 μ M (★) and 1 mM (▲) 3AB. Each point is the mean of at least 3 separate experiments in each of which trypanosomes were counted in duplicates.

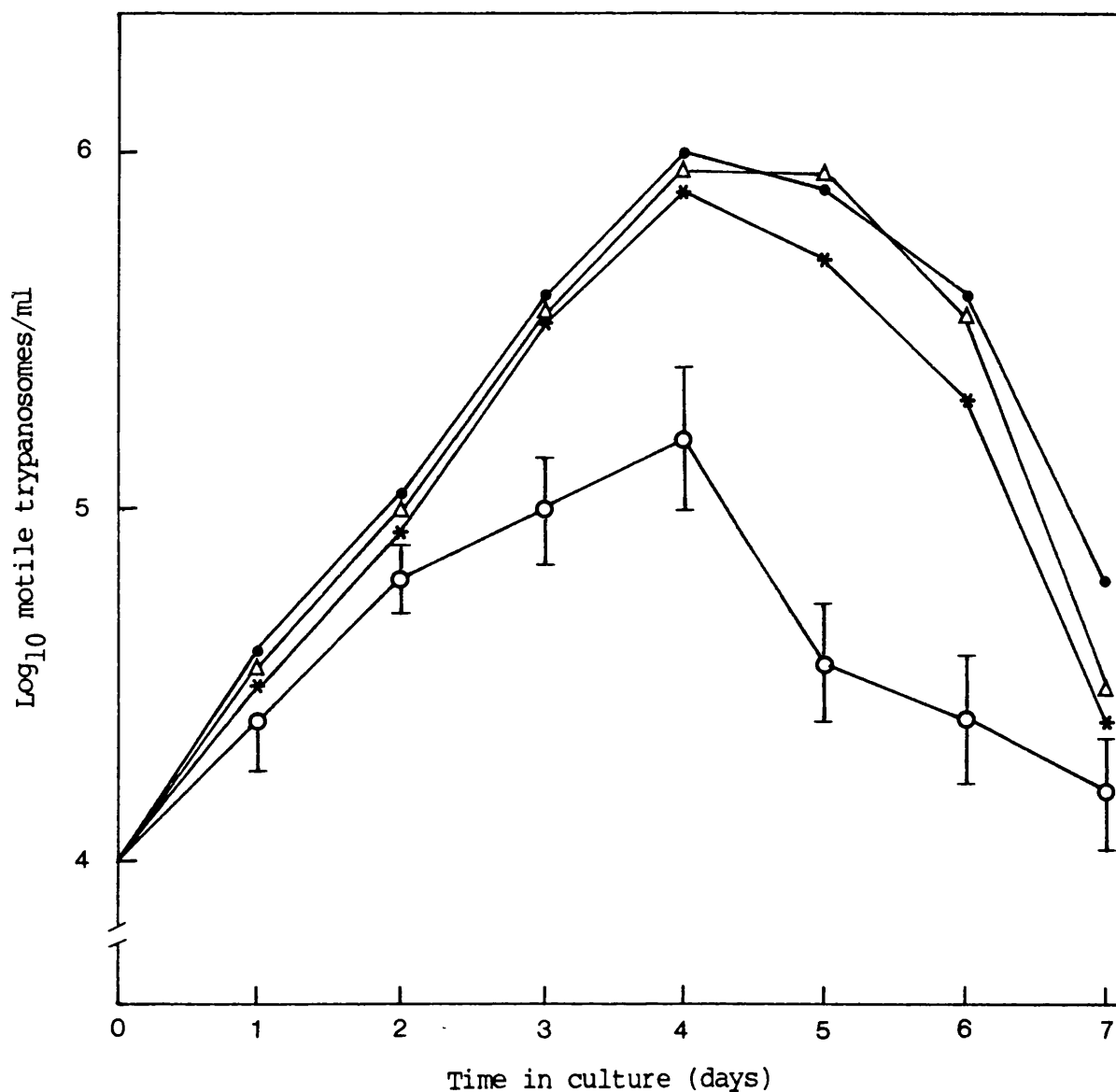


Fig. 6.2

The effect of 3AB on cultured T.brucei. Trypanosomes were cultured with an initial cell density of 1×10^4 trypanosomes/ml. 3AB was added to the cultures at the time of incubation. The number of viable trypanosomes were estimated by counting the motile organisms using haemocytometer and phase contrast microscopy. Control cells (●), 2 mM 3AB (Δ), 5 mM 3AB (*) and 10 mM 3AB (○). Each point is the mean of at least 3 separate experiments in each of which trypanosomes were counted in duplicates. The error bars have only been shown for the 10 mM 3AB concentration.

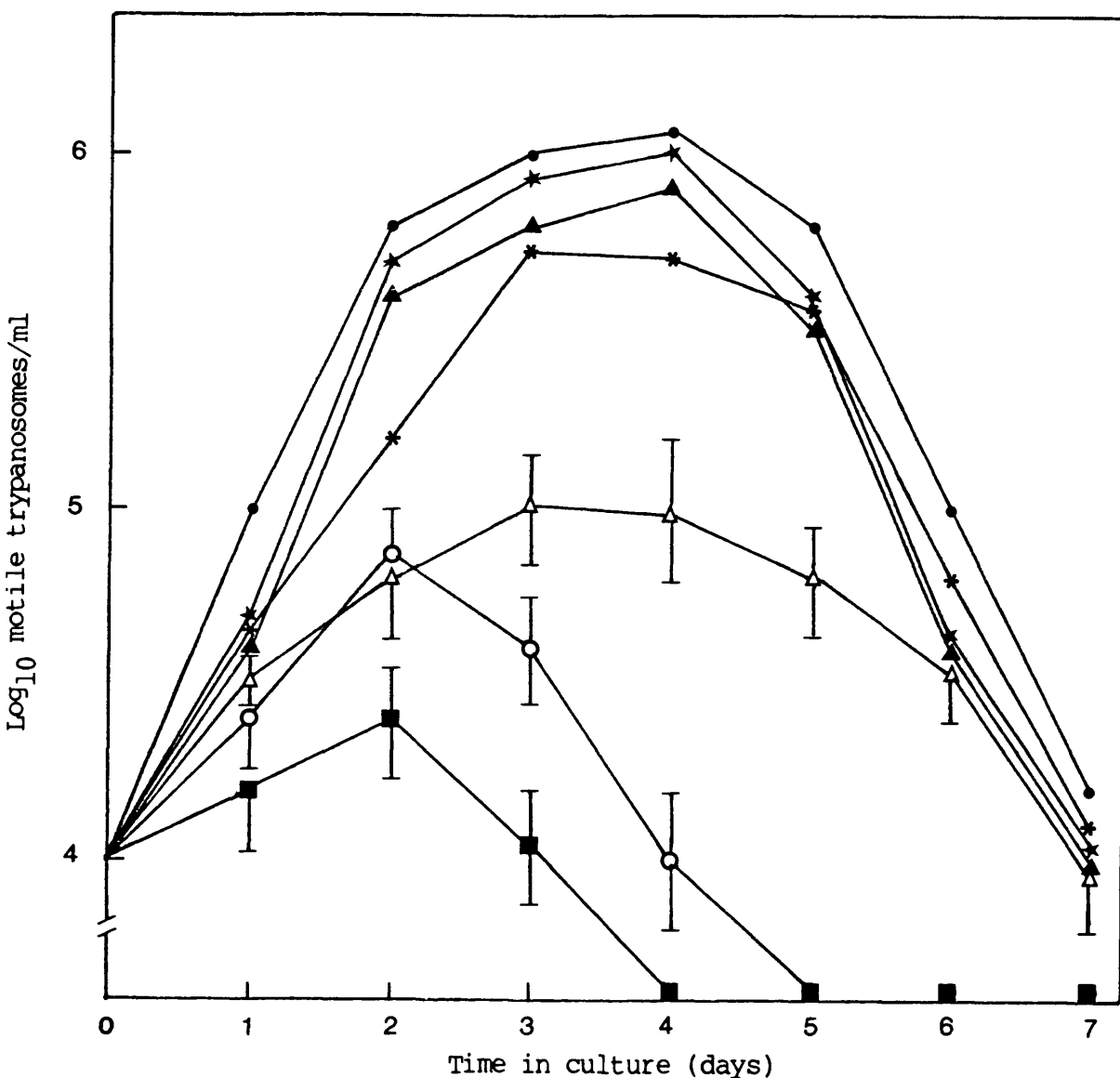


Fig. 6.3

The effect of 3AAB on cultured *T. brucei*. Trypanosomes were cultured at an initial cell density of 1×10^4 trypanosomes/ml. 3AAB was added to the cultures at the time of incubation. The number of viable trypanosomes were stimulated by counting the motile organisms using a haemocytometer and phase contrast microscopy. Control cells (●), 100 μ M (★), 200 μ M (▲), 500 μ M (*) 3AAB, 1 mM (Δ), 2 mM (○) and 5 mM 3AAB (■). Each point is the mean of at least 3 separate experiments in each of which trypanosomes were counted in duplicates. The error bars have only been shown for 1 mM, 2 mM and 5 mM concentrations of 3AAB.

order to investigate the enhancement of cytotoxicity following DNA damage induced by DMS and γ -radiation.

6.2.2 The Response of Cultured T.brucei to DMS and γ -radiation

In this section the effect of continuous in vitro treatment of T.brucei with various concentrations of DMS and also exposure to various doses of γ -radiation was studied. DMS was made up in 50% ethanol/H₂O (V/V) and was immediately added to the cultures to obtain the indicated concentrations. Control cultures were treated with the highest volume of the 50% ethanol (final ethanol concentration was 0.25%). Irradiation was performed at room temperature in a cobalt-60 source emitting approximately 0.5K rad/minute. Trypanosomes were exposed to approximately 0.5K rad, 1K rad, 2.5K rad, 5K rad, 7.5K rad and 10K rad of γ -radiation. Both DMS and γ -radiation treated trypanosomes were cultured at an initial density of 1×10^4 cells/ml.

Trypanosomes in all control cultures (untreated) increased in number and multiplied exponentially with a doubling time of 6-7 hours (Fig. 6.4). Treatment of trypanosomes with 5 μ M DMS also showed the same growth rate as control cultures. However when trypanosomes were treated with 10 μ M DMS the growth was retarded by a lag phase of about 48 hours (Fig. 6.4). In cultures treated with 20 μ M DMS, this lag phase was even larger. In these cultures the density slowly increased to 1×10^5 cells/ml in 5 days and 1×10^6 cells/ml after 10 days of culture (Fig. 6.4). 30 μ M DMS treatment caused a dramatic reduction in the culture density to approximately 200 cells/ml in 3 days. However a sharp increase in the trypanosomes population was observed in subsequent days with a growth rate comparable to that in untreated cultures. After 10 days of culture a density of 1.1×10^6 cells/ml was obtained (Fig. 6.4). In the 50 μ M DMS treated cultures

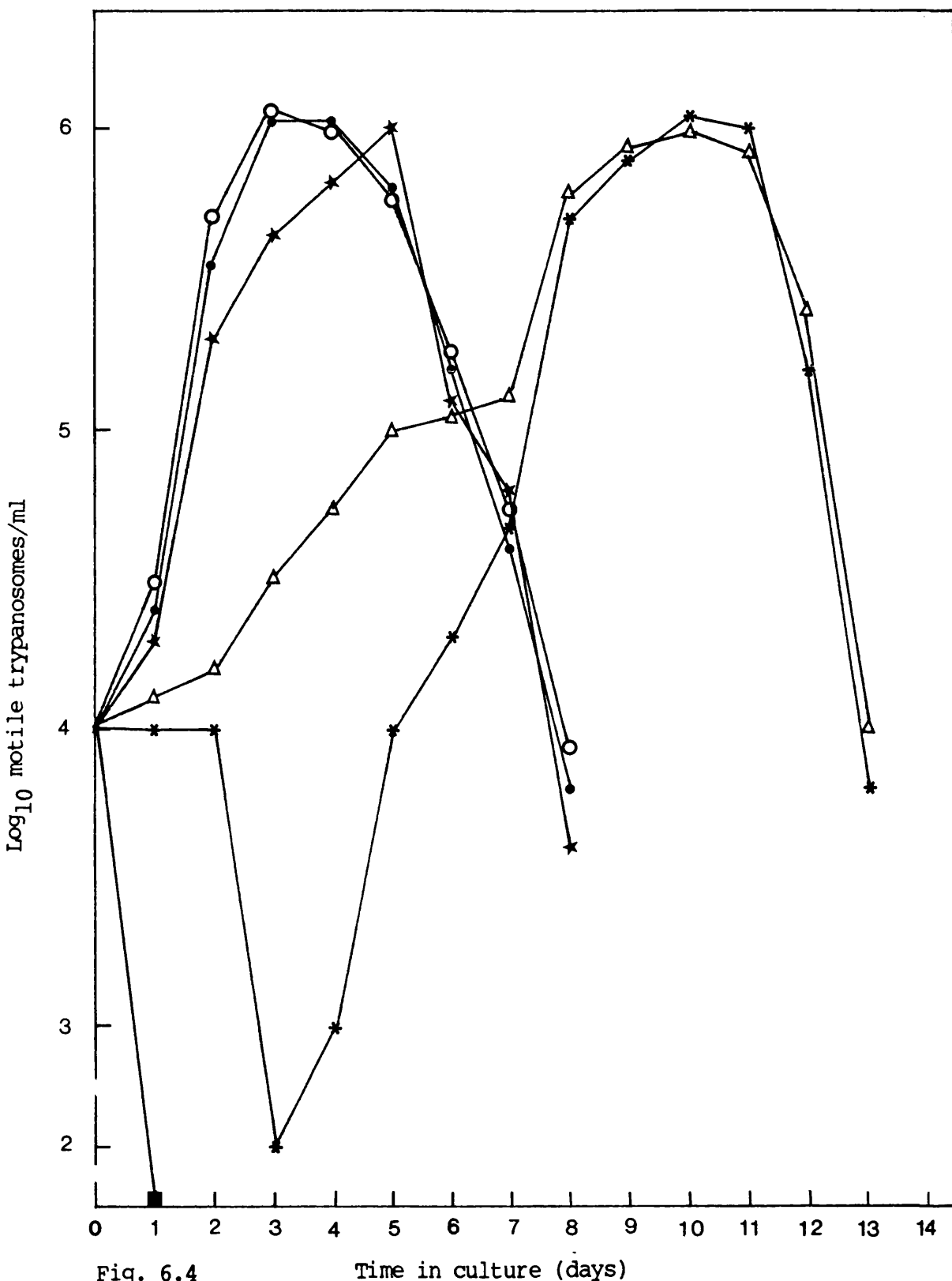


Fig. 6.4

Time in culture (days)

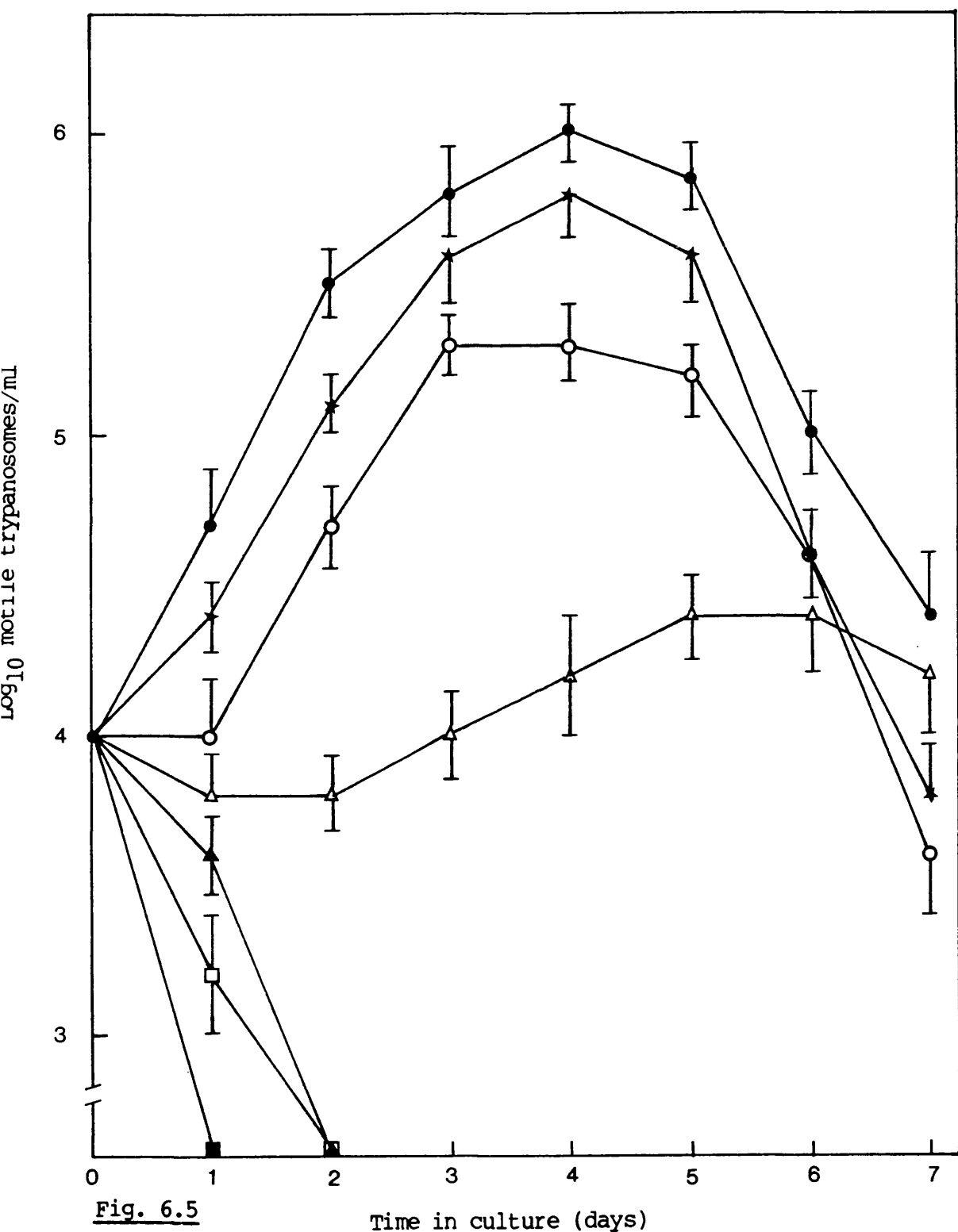
The effect of continuous DMS treatment on the survival and growth of *T. brucei*. Trypanosomes were cultured at an initial cell density of 1×10^4 /ml. DMS was added to cultures to the final concentrations of 5 μ M, 10 μ M, 20 μ M, 30 μ M, and 50 μ M. The number of viable trypanosomes were estimated by counting the motile organisms using a haemocytometer and phase contrast microscopy. control cells (O), 5 μ M (•), 10 μ M (*), 20 μ M (Δ), 30 μ M (x) and 50 μ M (■) DMS. Each point is the mean of at least 3 separate experiments in each of which trypanosomes were counted in duplicates.

there were no surviving trypanosomes (<1 in 10 mls of culture) within 24 hours of treatment. During the further incubation of these cultures for up to 7 days no viable trypanosomes were detected (Fig. 6.4).

Exposure of trypanosomes to 0.5K rad of γ -radiation (Fig. 6.5) results in reduction of cell density by approximately 10% (measured at 4 days post-irradiation). However the number of trypanosomes exposed to 1K rad of γ -radiation did not increase for the first 24 hours of culture, after which the density increased to approximately 2×10^5 cells/ml by 3-4 days after irradiation (Fig. 6.5). Exposure of trypanosomes to 2.5K rad of γ -radiation caused a decline in the number of viable trypanosomes during the first 48 hours. Even in these cultures the density subsequently increased to 2.5×10^4 cells/ml at 5 days, however the rate of growth was much slower than that in the control cultures; suggesting actual inhibition of growth as well as cell death. Higher doses of γ -radiation, 5, 7.5 and 10K rads resulted in a dramatic cell killing effect and cultures exposed to these doses of γ -radiation never recovered (Fig. 6.5).

6.2.3 The Enhancement of Cytotoxicity of DNA Damaging Agents by Inhibition of ADPR-transferase

The enhancement of the cytotoxic effect of DNA damaging agents (DMS and γ -radiation) on in vitro cultured trypanosomes by inhibition of ADPR-transferase activity by 3AAB was investigated. The reason for the choice of 3AAB as the inhibitor was that it has proven to be the most potent of the benzamide inhibitors group (Purnell and Whish, 1980). In this study 3AAB was used to test the role of ADP-ribosylation in the recovery of T.brucei from DNA damaging agents, DMS and γ -radiation.



The effect of γ -radiation on the survival and growth of *T. brucei*. Trypanosomes were exposed to various doses of γ -radiation. Irradiated trypanosomes were immediately cultured at an initial density of 1×10^4 cells/ml. The number of viable trypanosomes were estimated by counting the motile organisms using a haemocytometer and phase contrast microscopy. Control cells (●) and cells irradiated with 0.5 (★), 1 (○), 2.5 (Δ), 5 (▲), 7.5 (□) and 10 (■) K rads of γ -radiation. Each point is the mean of at least 3 separate experiments in each of which trypanosomes were counted in duplicates the bars indicate the standard error of the mean.

3AAB was added to the cultures at the concentration of 200 μM (the concentration at which there is no growth inhibition) 20-30 minutes prior to induction of DNA damage. This inhibitor was present throughout the experiments. This was to ensure that the inhibitor was present during any immediate repair after DNA damage thus acting on the entire process of DNA repair.

The appropriate volume of a solution of DMS, made up in 50% ethanol/ H_2O (V/V), was added to cultures to obtain final concentrations of 10 μM , 20 μM and 30 μM . Because the half-life of DMS in culture medium at 37°C is very short (approximately 15 minutes) the DMS was not washed away. γ -irradiation was performed at room temperature and trypanosomes were exposed to 0.5k rad, 1K rad and 2.5K rad of γ -radiation.

Trypanosomes treated with either DMS or γ -radiation were then cultured in T-25 flasks with 10 ml of medium/flask in the presence of Fisher rat embryonic fibroblasts. Incubation began with an initial cell density of 1×10^4 trypanosomes/ml of culture medium at 37°C in an atmosphere of 5% CO_2 and 95% air.

When 3AAB (200 μM) treated trypanosomes (20-30 minutes pre-incubated at 37°C) were exposed to 10 μM DMS growth was retarded by a lag phase of about 6-7 days, compared with trypanosomes in cultures treated with DMS only. The culture density then slowly increased to approximately 3×10^5 cells/ml in 11-12 days. Interestingly even after this time the maximum density was lower than that obtained in either control or DMS only treated cultures (Fig. 6.6). In the 3AAB treated cultures exposed to 20 μM DMS, cell density decreased to approximately 1×10^3 cells/ml in 48 hours after which a gradual increase at approximately the same rate as DMS only treated cultures

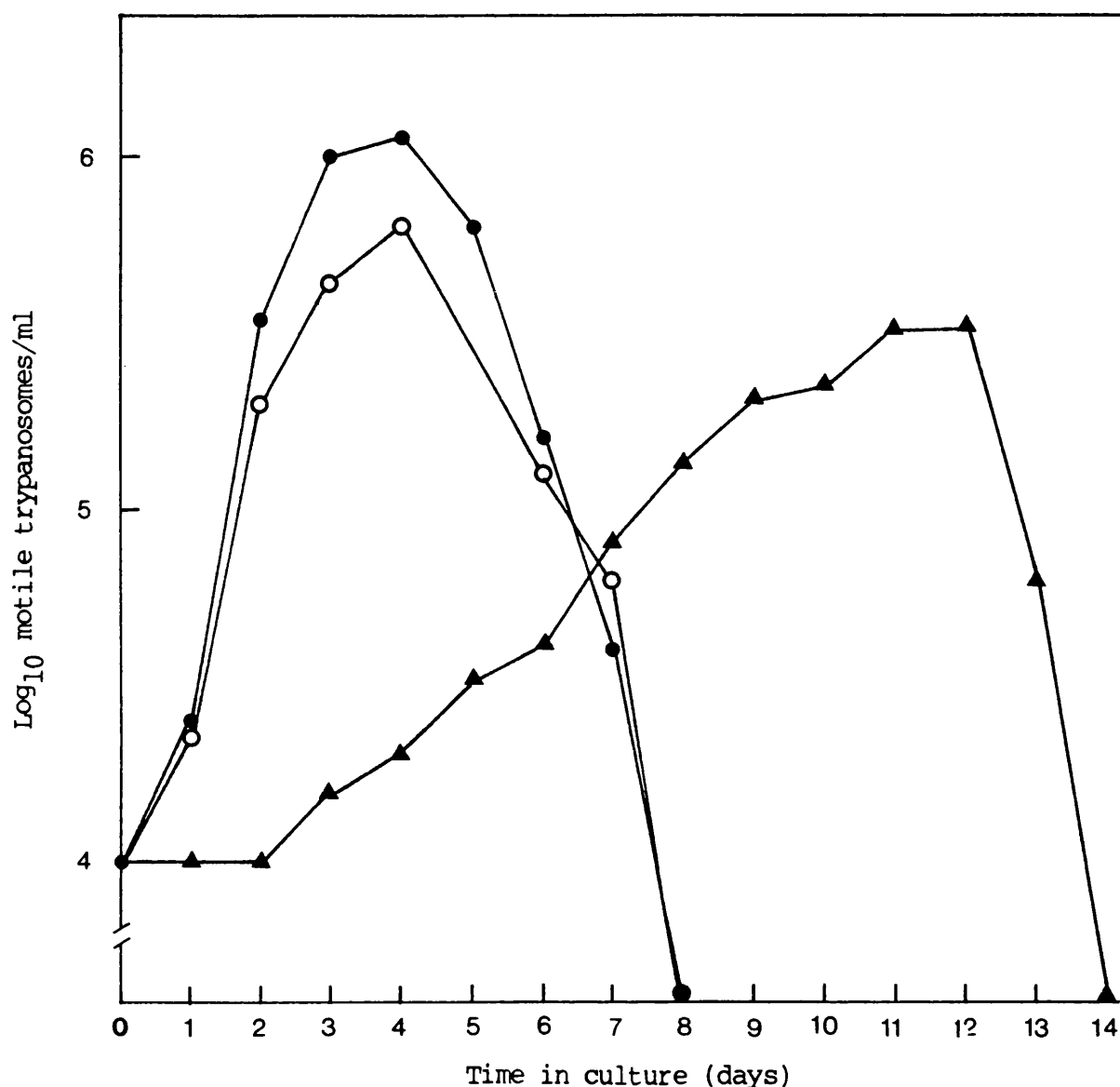


Fig. 6.6

The effect of DMS treatment on cultured T.brucei in the presence absence of 200 μM 3AAB. The number of viable trypanosomes we estimated by counting the motile organisms using a haemocytometer a phase contrast microscopy. Control cells (●), 10 μM DMS (○), 10 μM DMS + 200 μM 3AAB (▲). Each point is the mean of at least separate experiments in each of which trypanosomes were counted duplicates.

was observed. The maximum cell density (about 5×10^4 cells/ml) was again much lower than control or DMS only treated cultures and was obtained only after 13 days of culture (Fig. 6.7). Exposure of the 3AAB treated trypanosomes to $30 \mu\text{M}$ DMS resulted in the complete killing of all trypanosomes in culture by 48 hours. There was no evidence of recovery in these cultures up to 7 days of culture (Fig. 6.8).

Exposure of 3AAB treated trypanosomes to 0.5K rad of γ -radiation resulted in only a small reduction in the rate of growth and the maximum density obtained (Fig. 6.9, \star). However 3AAB treatment of trypanosomes exposed to 1K rad of γ -radiation resulted in a clear enhancement of cell killing as compared to either the control or only γ -irradiated cultures (Fig. 6.9, Δ). The density of trypanosomes in these cultures was reduced to approximately 5×10^3 cell/ml in 24 hours and then gradually increased to a maximum density of 2.5×10^4 cells/ml by 3-4 days after irradiation, compared to a maximum density of about 2×10^5 cells/ml in only irradiated cultures (Fig. 6.9, \blacktriangle). 2.5K rads of γ -radiation produced a high cell killing effect. Trypanosomes (pre-incubated with 3AAB) did not show any increase in numbers and all died within 6 days of γ -irradiation (Fig. 6.9, \square).

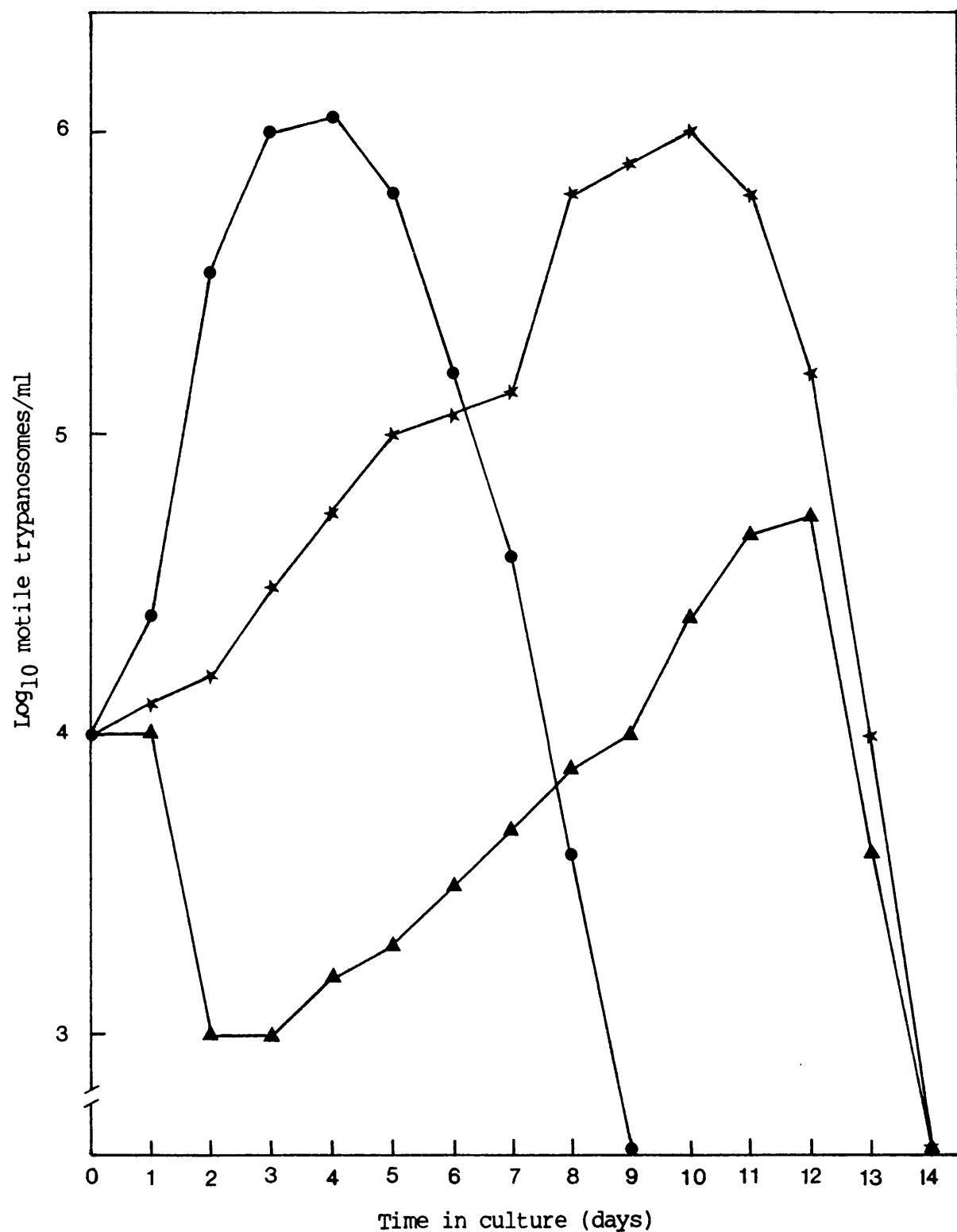


Fig. 6.7

The effect of 20 µM DMS treatment on cultured trypanosomes in the presence of 200 µM 3AAB. The number of viable trypanosomes were estimated by counting the motile organisms using a haemocytometer and phase contrast microscopy. Control cells (●), 20 µM DMS (★), 20 µM DMS + 200 µM 3AAB (▲). Each point is the mean of at least 3 separate experiments in each of which trypanosomes were counted in duplicates.

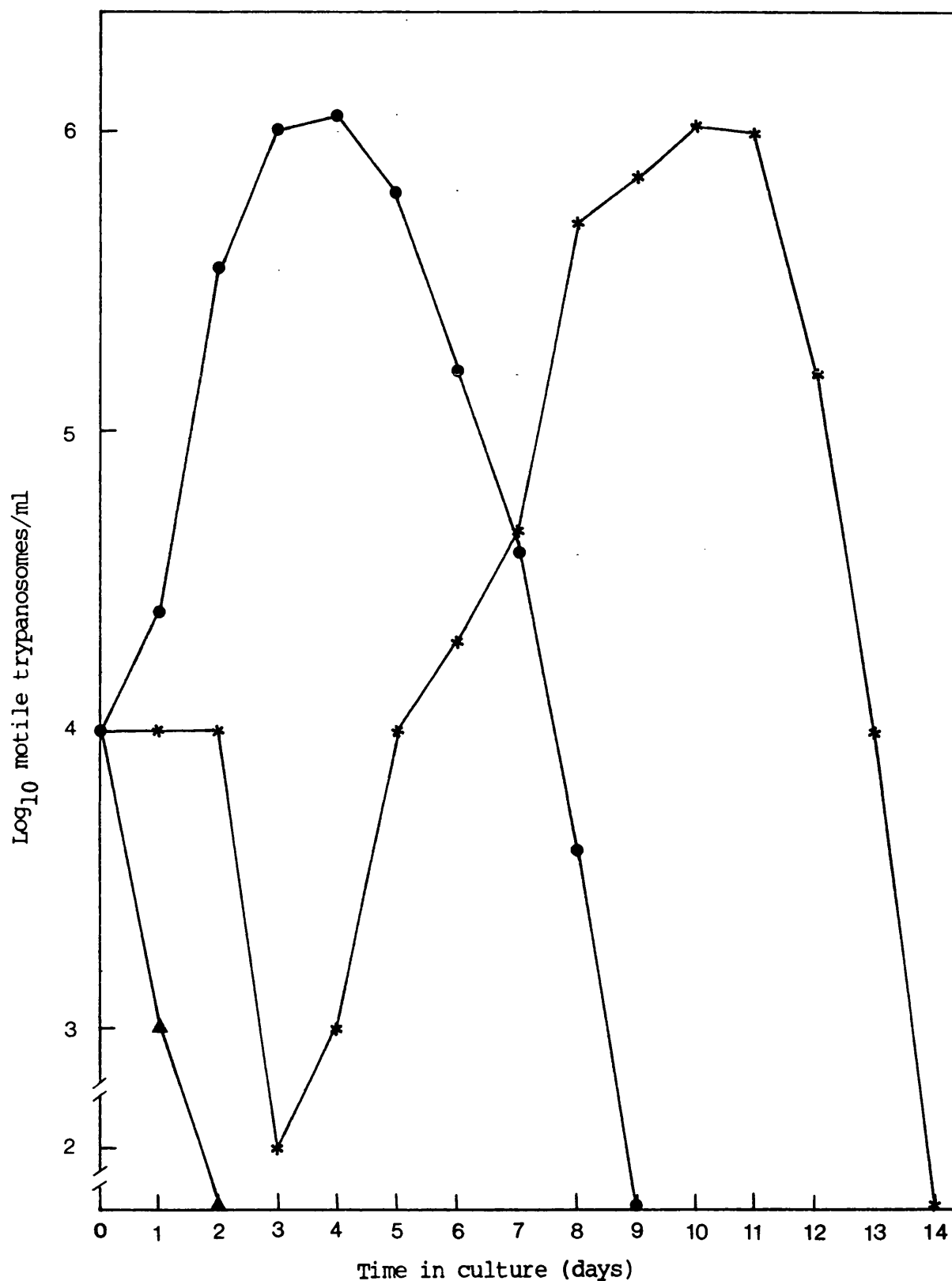


Fig. 6.8

The effect of 30 µM DMS treatment on cultured trypanosomes in the presence or absence of 200 µM 3AAB. The number of viable trypanosomes were estimated by counting the viable organisms using haemocytometer and phase contrast microscopy. Control cells (●), 30 µM DMS (*), and 30 µM DMS + 200 µM 3AAB (▲). Each point is the mean of at least 3 separate experiments in each of which trypanosome were counted in duplicates.

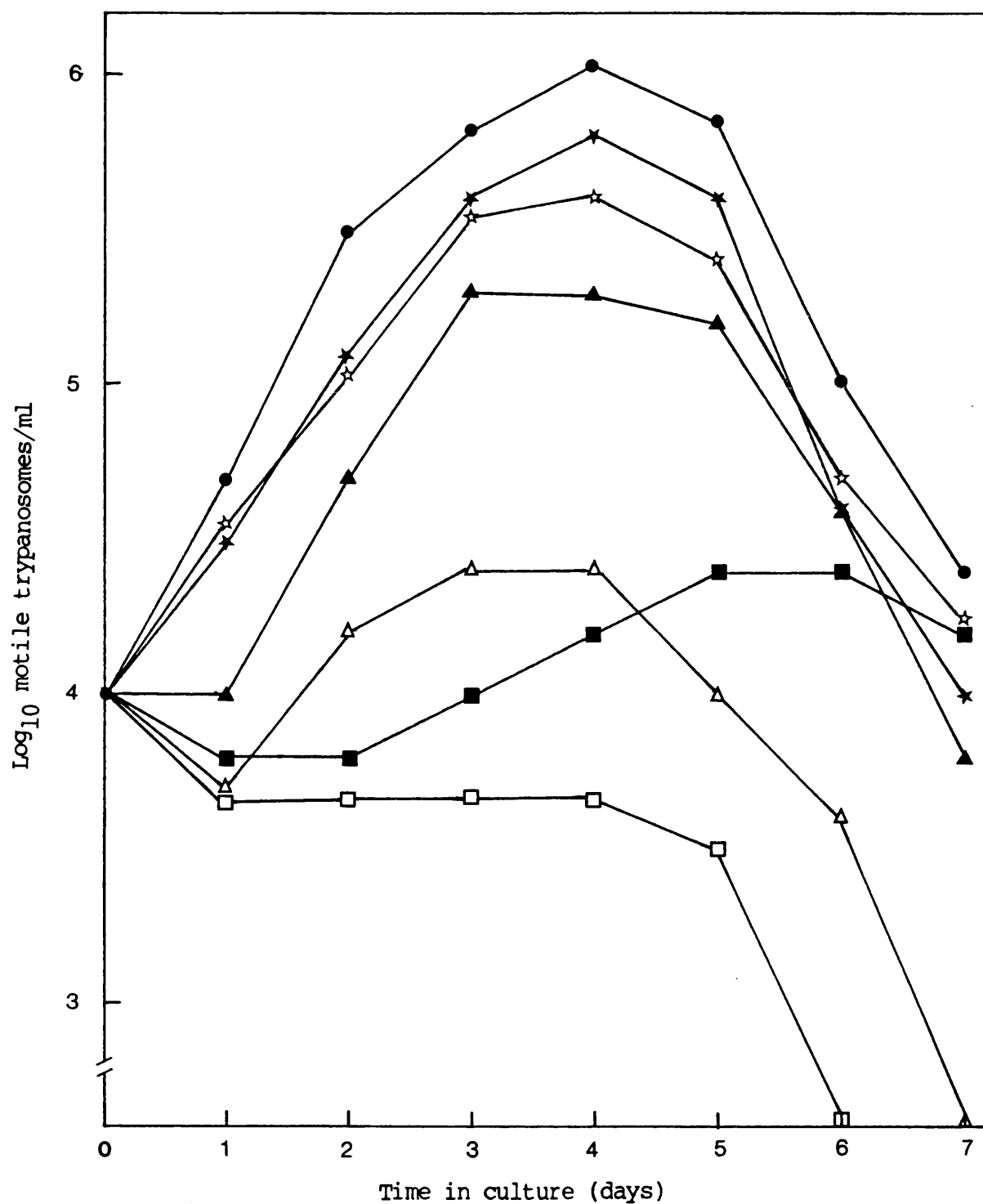


Fig 6.9

The effect of 0.5K rad of γ -radiation on cultured *T. brucei* in the presence or absence of 200 μ M 3AAB. The number of viable trypanosomes were estimated by counting the motile organisms using haemocytometer and phase contrast microscopy. Control cells (●), 0.5K rad γ -radiation (★), 0.5K rad γ -radiation + 200 μ M 3AAB (✱), 1K rad γ -radiation (▲), 1K rad γ -radiation + 200 μ M 3AAB (△), 2.5K rads γ -radiation (■), 2.5K rads γ -radiation + 200 μ M 3AAB (□). Each point is the mean of at least 3 separate experiments in each of which trypanosomes were counted in duplicates.

6.3 Discussion

In this study the highest non-toxic concentrations of the inhibitors of ADPR-transferase, 3AB and 3AAB in the continuous in vitro cultures of T.brucei were determined to be 2 mM and 200 μ M, respectively. Trypanosomes were found to be very sensitive to higher concentrations of these two inhibitors especially 3AAB. The effect of these non-toxic concentrations on the cytotoxicity and enhancement of cell killing of the DNA damaging agents, DMS and γ -radiation, were then determined.

Cultured trypanosomes exposed to DMS exhibited a dose dependent cytotoxic effect and at 50 μ M DMS no viable trypanosomes were detected after 24 hours post-incubation.

γ -irradiation of cultures also exhibited a dose dependent cell killing effect. The non-cytotoxic dose of γ -radiation was found to be about 0.5K rad. Higher doses of γ -radiation (1-10K rad) showed increasingly higher degrees of cell killing. However, it is interesting that when trypanosomes were exposed to up to 15K rads of γ -radiation the cellular NAD level recovered to control levels within about 15 minutes (see section 4.2.6). NAD is the substrate for ADP-ribosylation and by far the greatest proportion of its turnover is associated with this process.

Results indicate that in γ -irradiated trypanosomes the cellular NAD level is not an important indicator of the potential ability of the trypanosomes to survive and recover from the DNA damage.

Cytotoxicities of DMS and γ -radiation were both greatly enhanced by 3AAB (200 μ M). In the case of damage by alkylating agents the enhancement of cell killing by ADPR-transferase inhibition

has been attributed to the inhibition of DNA strand-break repair (Durkacz et al., 1980 & 1981; James and Lehman, 1982). Creissen and Shall (1982) suggested that this may be explained by the need for ADP-ribosylation to stimulate the DNA ligase activity necessary for the final step of DNA strand-break rejoining.

Previously reported studies had led to the conclusion that ADP-ribosylation has little or no role in the exposure of cells to ionising radiation. Davies et al. (1978) and Nduka et al. (1980) examined the effect of 2 mM 5-methylnicotinamide, 250 μ M theophylline and 250 μ M caffeine on mouse leukaemia L1210 cells and found these inhibitors to produce only a small increase in the cytotoxic effect of γ -irradiation. James and Lehman (1982) investigated the effect of 5 mM 3AB on the radiation response of human fibroblasts and found little or no enhancement of cell killing by γ -irradiation. They concluded that ADPR-transferase activity is unimportant for recovery of damage produced by γ -irradiation. However, Lunec et al. (1984), using 3AAB, demonstrated that ADPR-transferase activity is in fact required for recovery from radiation induced damage. This group showed significant enhancement of cell killing in the presence of ADPR-transferase inhibitors during the recovery period. They concluded that the necessary degree of inhibition can be achieved by a non-toxic concentration of 3AAB in the range of 2-4 mM and that the maximum effect is observed if the inhibitor is present during irradiation as well as several hours at 37°C post-irradiation.

Results presented in this section show that marked enhancement of cell killing by γ -radiation is observed when trypanosomes are treated with a non-toxic concentration of 3AAB (200 μ M).

CHAPTER SEVEN

In vitro Differentiation of T.brucei and
the Effect of ADPR-transferase Inhibitors

Results and Discussion

7.1 Introduction

The parasitic protozoan, T.brucei, undergoes a series of differentiation steps during its cyclical development in the mammalian host and the arthropod vector, the tsetse fly (Bowman et al., 1972; Brown et al., 1973; Bowman and Flynn 1976; Gitto et al., 1979 and Bienen et al., 1981). Differentiation is already initiated in the mammalian bloodstream when slender forms shift to stumpy forms. This phenomenon, known as pleomorphism, is closely associated with changes in oxidative metabolism. It has been demonstrated that morphological changes occurring during the life cycle of this parasite are accompanied by an alternating proliferation and regression of the mitochondrial structures, tricarboxylic acid cycle (TCA cycle) enzymes and cytochromes (Bowman et al., 1972; Hill, 1976; Ghiotto et al., 1979 and Bienen et al., 1981). In the slender multiplicative bloodstream forms of T.brucei the mitochondrion is reduced to a single peripheral canal and cristae are sparse or absent. These trypanosomes oxidize glucose by glycolysis to pyruvate which they can not degrade further. They also oxidize NADH via α -glycerophosphate oxidase system which is cyanide-insensitive.

Progression to the stumpy forms is marked by swelling of the mitochondrial canal, which develops tubular cristae. In stumpy forms some mitochondrial enzymes have been demonstrated but the TCA cycle is not yet fully functioning (Flynn & Bowman 1973 and Hill, 1976). During differentiation of bloodstream forms of T.brucei to procyclic forms, corresponding to midgut forms in the vector, the TCA cycle and terminal oxidation become fully functional (Brown et al., 1973; Hill, 1976 and Ghiotto et al., 1979). Stumpy forms can oxidise α -Ketoglutarate and, at a low rate, proline, suggesting some

mitochondrial participation in metabolism. The role of the kinetoplast in this differentiation is crucial, since mitochondrial energy metabolism is much more important to the procyclic forms than to the bloodstream forms (Bienen et al., 1981).

Experimentally induced differentiation of bloodstream trypanosomes to the procyclic stage can be initiated by transferring parasites from the blood of an infected mammal into tissue culture.

Since the most important change in energy metabolism during differentiation of bloodstream trypanosomes to procyclic forms is the activation of TCA cycle, it appeared reasonable to attempt stimulation of differentiation by directly activating the TCA cycle. The observation of Brun and Schonenberger (1981), that citrate and/or cis-aconitate stimulates differentiation led to the suggestion that these metabolites serve as activators or inducers of isocitrate dehydrogenase and/or other TCA cycle enzymes. This switch therefore acts as a metabolic trigger for the morphological changes involved in differentiation. Czichos et al. (1986) suggested that in fact synchronous differentiation of the bloodstream forms of the variant MITat 1.4 to the dividing procyclic cells requires the combined action of two signals: the addition of citrate/cis-aconitate (3mM each or of 6mM cis-aconitate) as inducers and a decrease in the temperature from 37°C to 27°C.

In this section the aim was to establish a reproducible culture system that allows morphological differentiation of monomorphic bloodstream forms of T.brucei (Strain MITat 1.1) to procyclic cells.

7.2 Results

7.2.1 In vitro differentiation of bloodstream forms of T.brucei to procyclic forms

Freshly isolated bloodstream forms of the variant MITat 1.1 were cultured with an initial cell density of 5×10^5 cells/ml of SDM-79 medium (see materials 3.1.9d). This medium contained 10% (V/V) fetal calf serum.

The presence of the feeder cells, Fisher rat embryonic fibroblasts (see materials 3.1.8d), throughout the entire differentiation process was also found to be essential. Where indicated, the required volume from a stock solution of cis-aconitate (30 mM), to give a final concentration of 3 mM, was added to the cultures. Fig. (7.1) demonstrates representative results from experiments that were repeated several times with similar results. Cells were counted every 24 hours by phase contrast microscopy in a haemocytometer at 400x magnification. Distinction between bloodstream trypanosomes and differentiated cells were made at the time of counting, using the following criteria; size, shape, motility, and presence or absence of undulating membranes. SDM-79 medium did not support either growth or maintenance of the bloodstream forms of trypanosomes at 37°C. In the presence of 3 mM cis-aconitate, at 37°C, the parasite number decreased rapidly from 5×10^4 to 1×10^4 cells/ml. All the organisms in culture died within 72 hours if incubation was prolonged at this temperature (Fig. 7.1, ★). Upon transfer from 37°C to 27°C the cells responded in two ways. Firstly, the majority retained the morphology of bloodstream forms and did not grow for at least 24 hours. Upon prolonged incubation most of these cells disintegrated. Secondly, a small fraction

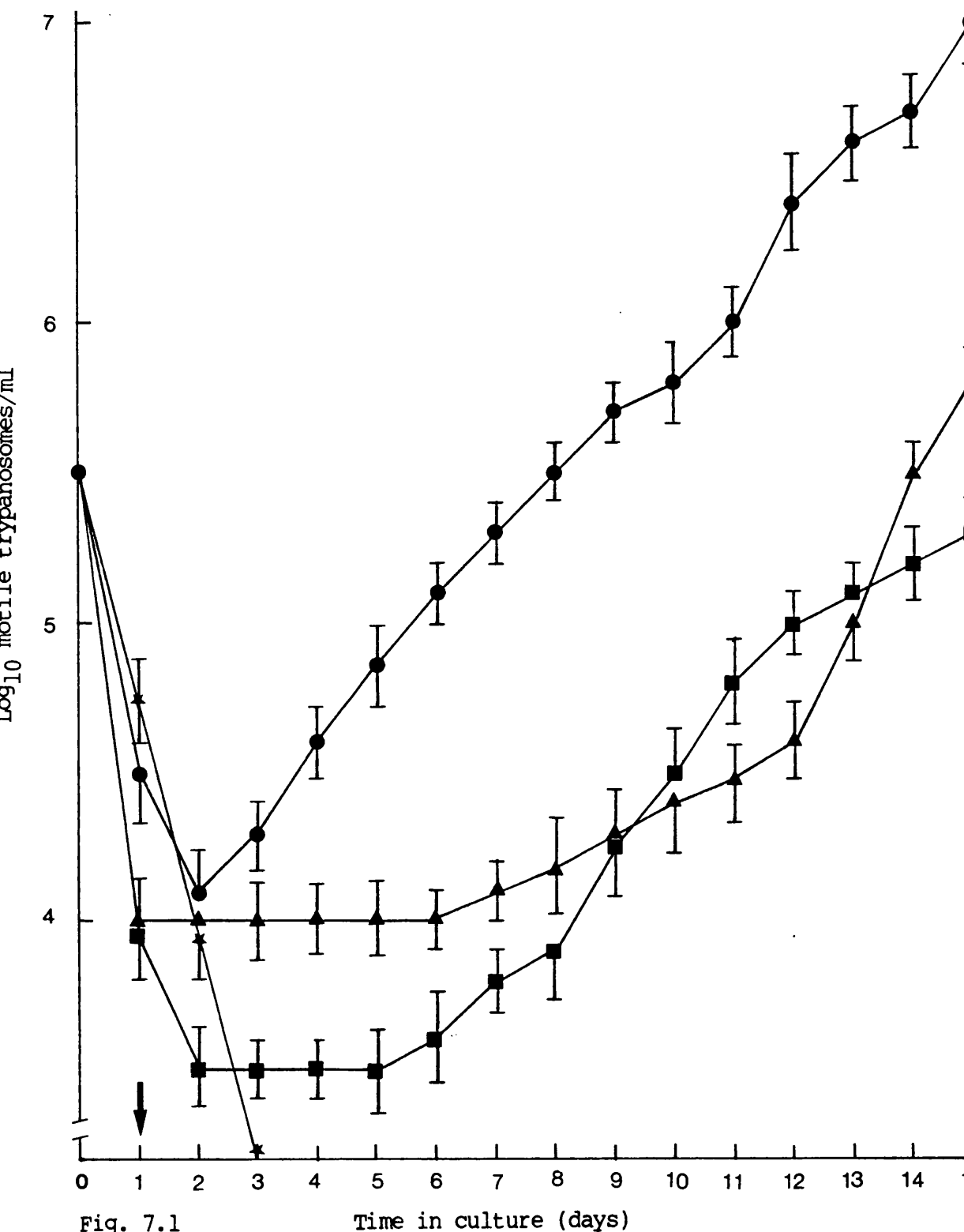


Fig. 7.1

Time in culture (days)

Effect of a temperature change in the presence or absence of cis-aconitate on differentiation of *T. brucei*. SDM-79 medium supplemented with 10% (V/V) fetal calf serum was used for all the cultures. Overnight pre-adapted bloodstream trypanosomes at 37°C were transferred to 27°C in the presence of cis-aconitate (●), in the absence of cis-aconitate (■), or directly incubated at 27°C (without the period of pre-adaptation) in the presence of cis-aconitate (▲) and continuous incubation at 37°C in the presence of cis-aconitate (★). The arrow indicates the time of transfer of cultures from 37°C to 27°C. Each point is the mean of at least 4 separate experiments in each of which trypanosomes were counted in duplicates. The bars indicate the standard error of the mean.

differentiated successfully and then divided (Fig. 7.1, ●). The period of pre-adaptation (overnight incubation at 37°C prior to transfer to 27°C) was found to be necessary since the direct incubation of trypanosomes at 27°C in the presence of cis-aconitate (3 mM) delayed the process of differentiation for 6 days (Fig. 7.1, ▲). In the absence of cis-aconitate however, the differentiation was still initiated but was delayed for the slightly shorter time of approximately 5 days (Fig. 7.1, ■). In this system, complete morphological differentiation of T.brucei MITat 1.1, as observed by light microscopy using the above mentioned criteria, was complete in 2 days (Fig. 7.1, ●) in the presence of 3 mM cis-aconitate and 5 days in the absence of this TCA cycle intermediate (Fig. 7.1, ■).

Bloodstream forms grown overnight at 37°C in the presence of cis-aconitate (3 mM) were similar to a pleomorphic population containing a high proportion of stumpy forms. Semi-synchronous differentiation to procyclic cells occurred when these cultures were transferred to the temperature of 27°C. Cell growth showed a sigmoidal-type curve and the primary culture could be maintained without further sub-culturing for up to 2 weeks reaching a maximum density of 1×10^7 cells/ml.

Bienen et al. (1981) suggested that biochemical differentiation occurs on a different time scale compared to morphological differentiation. His group showed that differentiating cells do not attain a normal respiratory pattern typical of established procyclic trypomastigotes (70-80% cyanide sensitive, 20-30% SHAM sensitive) until 20 to 24 days after inoculation into culture. Overath et al. (1986) showed that when differentiation is triggered at 27°C in the presence of citrate/cis-aconitate, the rapid decrease in VSG

synthesis is followed by the development of mitochondrial functions in the newly differentiated procyclic cells. They showed that after 72 hours the rate of respiration of most substrates is close to that of established procyclic cells. At this time only 25% of the total rate of respiration of glucose is inhibited by salicylhydroxamic acid (SHAM) while 75% is cyanide-sensitive. It is therefore evident that morphological and biochemical differentiation may take place at different times.

Development of this system facilitated the study of the involvement of ADPR-transferase activity in the in vitro differentiation of bloodstream forms of T.brucei to procyclic forms. The fully differentiated trypanosomes, as visualised by phase contrast microscopy, were maintained for up to 3 months in the absence of feeder cells. The cultures would normally reach a cell density of 1×10^7 procyclic trypanosomes/ml. However, they were usually subcultured in the late log phase when the culture density was approximately 5×10^6 cells/ml. Injection of these T.brucei, experimentally induced to differentiate from the bloodstream forms to the procyclic forms, to female Wistar rats did not cause any infection. In these experiments four rats were inoculated each with 10^7 procyclic forms which had been isolated from four different cultures in the log growth phase. The induction of differentiation into procyclic forms was also confirmed by the lysis of the trypanosomes from these cultures by exposure to human serum (see Chapter eight, Appendix, for details).

7.2.2 Effect of ADPR transferase Inhibitors on Morphological Differentiation of T.brucei

The presence of the enzyme ADPR-transferase and its involvement

in the differentiation of T.cruzi has already been reported (Williams, 1983b & 1984). Williams demonstrated that competitive inhibitors of ADPR-transferase specifically inhibited both intracellular and the extracellular differentiation of T.cruzi.

The culture system developed to study the differentiation of bloodstream forms of T.brucei to procyclic cells (see section 7.2.1) was used to investigate the possible involvement of ADPR-transferase activity in the differentiation of these organisms. The competitive inhibitors of ADPR-transferase, 3-aminobenzamide (3AB) and 3-acetamidobenzamide (3AAB) were used in these studies.

The experiments were carried out in T-25 flasks with 10 ml of culture medium/flask. Incubation began with an initial cell density of 5×10^5 freshly isolated trypanosomes/ml of SDM-79 medium in the presence of Fisher rat embryonic fibroblasts. SDM-79 medium was supplemented with 10% (V/V) fetal calf serum. The required volume from a stock solution of (30 mM) cis-aconitate was added to all the cultures to obtain a final concentration of 3 mM. Inhibitors were also added at the indicated concentrations and maintained continuously in the culture. All cultures were incubated overnight at 37°C in an atmosphere of 5% CO₂ and 95% air and then transferred to 27°C.

In this system bloodstream forms of T.brucei differentiated to procyclic forms. After about 14 days at 27°C the number of differentiated procyclic forms trypanosomes increased to approximately 10^7 cells/ml. Competitive inhibitors of ADPR-transferase 3AB and 3AAB both substantially reduced the number of procyclic trypanosomes to approximately 10^6 and 10^5 cells/ml in 2 mM and 5 mM 3AB, respectively (Fig. 7.2); and to approximately 2×10^5 and

2×10^4 cells/ml in 200 μ M and 500 μ M 3AAB, respectively (Fig. 7.3). These results indicate that inhibition of ADPR-transferase activity by the competitive inhibitors, 3AB and 3AAB, at non-toxic concentrations, blocks the induction of morphological differentiation of bloodstream forms of T.brucei to procyclic forms. At the same concentrations of inhibitors, 3AB and 3AAB, however, proliferation of bloodstream trypanosomes was not affected (see section 6.2.1).

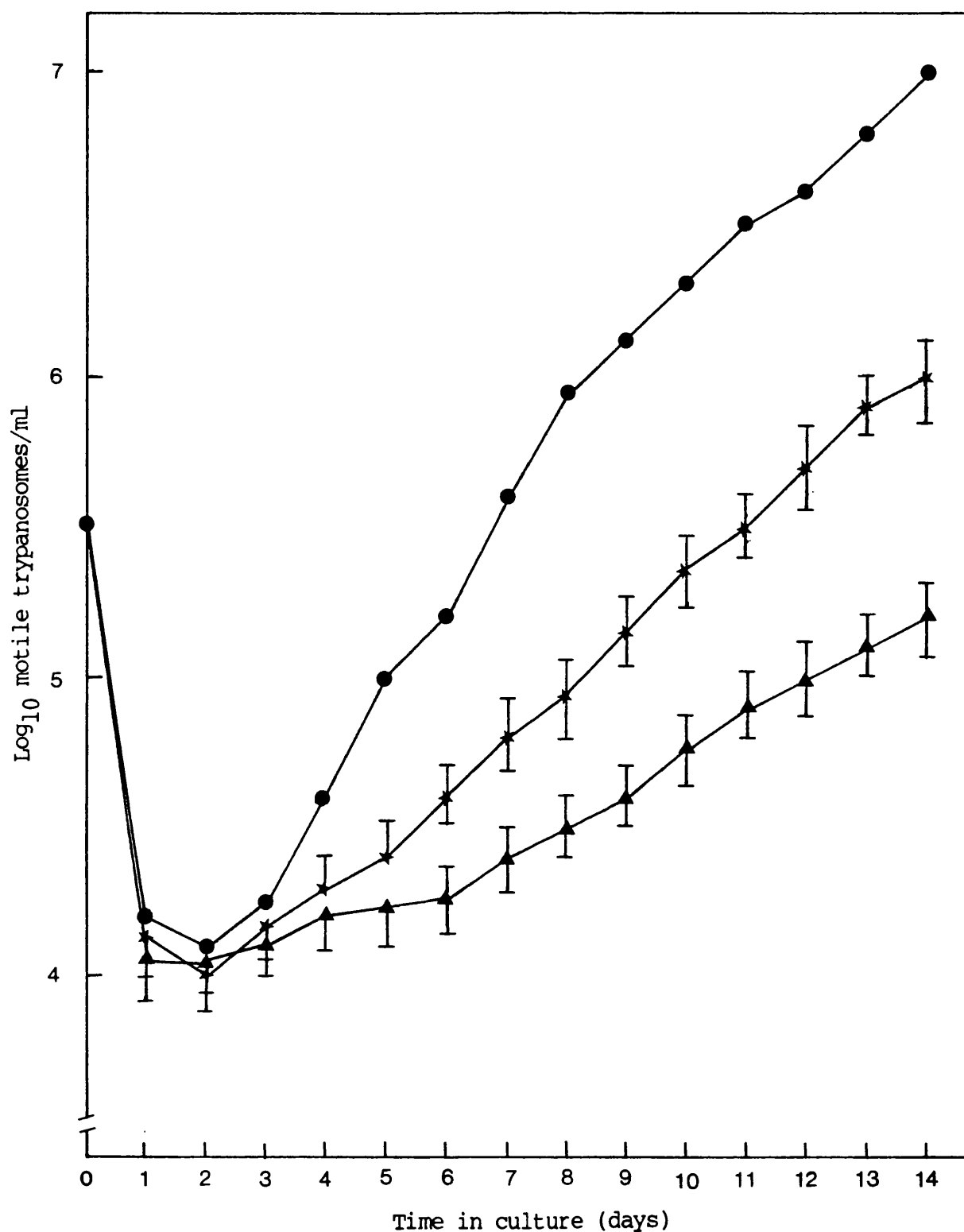


Fig. 7.2

Effect of ADPR-transferase inhibitor (3AB) on morphological differentiation of *T. brucei*. All cultures were incubated overnight at 37°C in the presence or absence of 3AB and also in the presence of 3 mM cis-aconitate. Cultures were then transferred to 27°C in the continued absence (●) or presence of either 2 mM (★) or 5 mM (▲) 3AB. Each point is the mean of at least 4 separate experiments in each of which trypanosomes were counted in duplicates. The bars indicate the standard error of the mean.

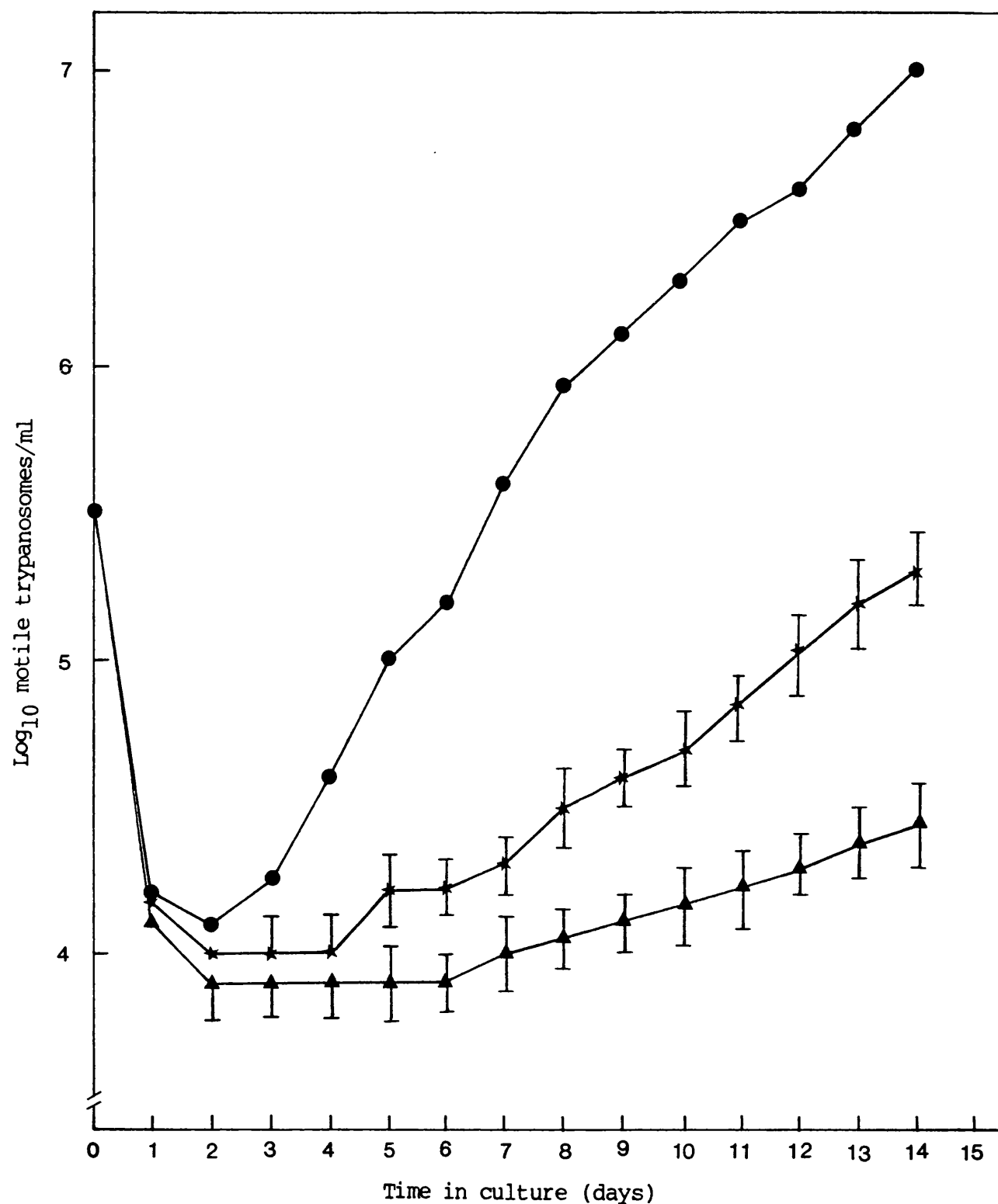


Fig. 7.3

Effect of ADPR-transferase inhibitor (3AAB) on morphological differentiation of *T. brucei*. All cultures were incubated overnight at 37°C in the presence or absence of 3AAB and also in the presence of 3 mM cis-aconitate. Cultures were then transferred to 27°C in the continued absence (●) or presence of either 200 μM (★) or 500 μM (▲) 3AAB. Each point is the mean of at least 4 separate experiments in each of which trypanosomes were counted in duplicates. The bars indicate the standard error of the mean.

7.3 Discussion

Several different studies have suggested that ADPR-transferase may play a role in cellular differentiation. In higher eukaryotes the differentiation events are usually effectively irreversible, whereas in protozoa they are cyclical. Both types of cell differentiation are more likely to result from changes which occur early in the sequence of events required for the expression of genetic information.

Many of the species in the kinetoplastida, which are of considerable importance as the causative agents of human or animal diseases, are heavily dependent on morphological differentiation during their infection cycles. In protozoa, cell differentiation results in a series of transformations through morphologically distinct stages in a cycle which eventually reproduces the original form of the organism. These transformations can now be simulated in vitro. T.cruzi for example, differentiates between amastigote, and epimastigote forms during its life cycle in vertebrate and invertebrate hosts (Brener, 1973 & 1981). Experimentally induced differentiation of bloodstream forms of T.brucei to the procyclic stage has also been extensively studied. Despite the fundamental importance of cell differentiation in the biology of eukaryotes, the understanding of the mechanisms controlling this process is very limited. However, several reports have suggested that modification of proteins by ADPR-transferase is required for cell differentiation (see chapter one). It has also been reported that competitive inhibitors of ADPR-transferase block the differentiation of chick myoblasts (Farzaneh et al., 1980 & 1982), the mitogen-induced activation of human peripheral blood lymphocytes (Johnstone and Williams, 1982), the induction of foetal enzymes in cultured

hepatocytes (Althaus et al., 1982) and intracellular and extracellular differentiation of T.cruzi (Williams, 1983b & 1984). These reports provided the first clear evidence that ADPR-transferase activity is widely required in the differentiation of higher animals.

The results presented in this work demonstrate that bloodstream forms of the monomorphic variant clones of the MITat 1.1 undergo morphological differentiation at 27°C in the presence of 3 mM cis-aconitate. Morphological differentiation of bloodstream trypanosomes to procyclic forms occurred within 24 hours after the transfer of preadapted bloodstream trypanosomes from 37°C to 27°C. The continued presence of the mammalian feeder cells (for at least 48 hours) was required for optimal differentiation without cell death. In addition, the continued presence of cis-aconitate (3 mM) was highly beneficial, both in increasing the rate of differentiation and in decreasing cell death in the early stages. Cell division does not occur until the later stage of the differentiation process (48 hours) and seems to be confined to newly formed procyclic cells. This work demonstrates that cis-aconitate (3 mM) can be of great practical use in experiments where rapid differentiation of bloodstream monomorphic trypanosomes is required.

As with T.cruzi (Williams, 1983b & 1984) morphological differentiation in T.brucei can be inhibited by benzamide derivatives, the competitive inhibitors of ADPR-transferase activity. The inclusion of these inhibitors (3AB & 3AAB) in the cultures produced a dramatic reduction in the number of procyclic trypanosomes.

Although the effect of ADPR-transferase inhibitors on the growth of the experimentally differentiated procyclic trypanosomes were not

directly studied (due to lack of time), the inhibitory effect is unlikely to have been caused by inhibition of proliferation (see section 6.2.1). The compounds therefore appear to affect an aspect of cellular metabolism which is involved specifically in the process of differentiation, as also seems to be the case in the cells of higher animals (Farzaneh et al., 1980 & 1982 and Johnstone & Williams, 1982). The inhibition of T.brucei differentiation in this experimental system, by inhibitors of ADPR-transferase, strongly suggests that the enzyme activity is required for the differentiation of these organisms.

In order to confirm the specificity of the effect of ADPR-transferase inhibitors to the inhibition of differentiation and not growth it would be highly desirable to study the effect of exposure to these inhibitors during short intervals (eg. 24 hours) at the start of differentiation. Correspondingly, exposure of the fully differentiated cultures to the inhibitors (eg. after 1 week) would determine whether in fact the growth of the already differentiated trypanosomes could be affected by the inhibition of ADPR-transferase activity. These would be important experiments enabling a clear distinction between the effect of the inhibitors on differentiation and their possible effects on the growth of the procyclic trypanosomes.

CHAPTER EIGHT

APPENDIX

Human Serum: A Tool to Assess the Induction of Differentiation
in T.brucei from Bloodstream Forms to Procyclic Forms

8.1 Introduction

Host range among trypanosomes of the brucei subgroup of African trypanosomes appears to be dependent in part on the cytotoxic properties of normal (non-immune) serum of the mammalian host. The lytic effect of serum is not dependent on complement and exhibits specificity with regard to both host and parasite (Rifkin, 1978). Thus, serum from permissive hosts (eg. rats, rabbits) is not cytotoxic for T.brucei whereas serum from non-permissive hosts (eg. humans, baboons) lyses these trypanosomes. The toxic effect of normal human serum on T.brucei was first described by Laveran (1902). Since that time, many reports (York et al., 1930; Rickman & Robson, 1970 and Hawking, 1973) have appeared on the selective lytic properties of human serum on the morphologically indistinguishable trypanosomes of the brucei group. The factor in human serum responsible for selective lysis of T.brucei has been identified as high-density lipoprotein (HDL) (Rifkin, 1978). Rifkin reported that the trypanocidal properties of human HDL were identical to those of unfractionated serum. This finding was confirmed by the demonstration that serum from patients with Tangier disease, an autosomal recessive disorder characterized by a severe lack of HDL (Frederickson et al., 1972), had no in vivo or in vitro lytic effect on T.brucei (Rifkin, 1978). It has been suggested that the sensitivity of trypanosomes to serum lysis may be dependent on the particular variant surface glycoprotein (VSG) present on the surface of the parasite (Van Meirvenn et al., 1976). Trypanosomes are coated with about 10^7 molecules of this single glycoprotein, (Cross, 1975 & 1978) which protect them from the mammalian host's immune system.

In tissue culture experiments, Stein and Stein (1973) found that HDL was capable of removing cholesterol from arterial smooth muscle

cells. Although cholesterol removal in these and other cells is probably minimal, excessive cholesterol removal in erythrocytes can lead to increased membrane permeability, osmotic fragility, and eventually lysis (Bruckdorfer et al., 1969). HDL apoproteins have also been shown to remove phospholipids from Landshutz ascites cells (Jackson et al., 1975). The finding that lysis of trypanosomes by normal human serum is the result of acute damage to the membrane permeability properties of the parasite is consistent with the hypothesis that the interaction of human HDL with the surface of T.brucei may result in a lethal alteration in the lipid composition of the plasma membrane (Rifkin, 1978). Rifkin (1978) suggested that the extent of damage might depend on the magnitude of the difference between the cholesterol to phospholipid ratio of HDL and the cholesterol to phospholipid ratio of the plasma membrane of trypanosomes. The cholesterol to phospholipid ratio of HDL from different hosts would then determine the extent of cellular lipid removal among trypanosomes of a given strain. Thus HDL from a permissive host (rat) would be expected to remove relatively less lipid from T.brucei than HDL from a restrictive host (human). Likewise, it is possible that differences in the cholesterol to phospholipid ratio of the plasma membranes of T.brucei and T.rhodesiense would influence the effect of human HDL on these two species.

Alternatively, the relative resistance of T.rhodesiense to lysis by HDL might be due to the enhanced ability of these trypanosomes to repair the membrane lesion caused by HDL.

The differentiation of the bloodstream trypomastigote forms of T.brucei into the procyclic culture forms, which survive in the tsetse midgut, involves some external morphological changes combined

with several dramatic internal ultrastructural and physiological changes. One of these changes is the loss of the surface coat.

Considering the specific lysis of bloodstream forms of T.brucei by normal human serum as a result of interaction between the serum HDL and trypanosome surface it was reasonable to assume that since procyclic forms of T.brucei lack the surface protein coat they might be much more susceptible to lysis when exposed to human serum. The possibility of using the differential lysis of the bloodstream and procyclic forms, by HDL, as a tool for the identification of the two forms of T.brucei in culture was investigated.

8.2 Materials and Methods

8.2.1 Materials

8.2.1.1 T.brucei, bloodstream forms

T.brucei (Strain MITat 1.1) bloodstream forms were purified from the blood of infected Wistar rats (see section 3.2.1.8).

8.2.1.2 T.brucei, procyclic forms

a- Experimentally induced differentiation of T.brucei were used from different cultures.

b- Procyclic forms of T.brucei, established in culture from an inoculum taken from the midgut of tsetse fly, were provided by Dr. W.Gibson, Dept. of Pathology, University of Bristol.

8.2.2 Methods

8.2.2.1 Preparation of Sera

Normal human blood was obtained from healthy (presumably non-

immune) human volunteers by venipuncture. The samples were left overnight to clot. Appropriate volumes of the serum were used for trypanocidal assays. Rabbit serum was prepared as described in section (3.2.1.2). Heat inactivated fetal calf serum was obtained from Gibco.

8.2.2.2 Assay for trypanocidal activity

Standard in vitro incubation conditions were 37°C for bloodstream forms and 27°C for procyclic forms at a cell density of approximately 5×10^6 trypanosomes/ml of culture medium. For bloodstream forms of T.brucei the culture medium was DMEM, control tubes were supplemented with 25% (V/V) rabbit serum and experimental tubes contained 25% (V/V) human serum. The culture medium for procyclic forms was SDM-79, control tubes contained 25% fetal calf serum, alternatively for the trypanocidal assays various concentrations of human serum (5-25% V/V) were added. Both media contained 2% glucose. The number of intact trypanosomes remaining after incubation were counted using a haemocytometer. Fraction of lysed trypanosomes were determined by subtraction of the number of intact trypanosomes remaining from the number present at the start of incubation.

8.3 Results

8.3.1 Effect of Normal Human Serum on T.brucei

When bloodstream forms of T.brucei were treated with 25% (V/V) of unfractionated human serum the actual lysis was preceded by a lag phase of about 30 minutes after which the complete lysis of all trypanosomes in the incubation mixture took approximately 3 hours. However trypanosomes in medium (DMEM) containing 25% rabbit serum

were very active and no trypanocidal activity was observed over the same periods of time (Fig. 8.1).

By contrast, human serum showed a dramatic trypanocidal effect on established procyclic forms of T.brucei at all concentrations used, 5-25% (V/V). The lysis of the established procyclic trypanosomes began immediately after incubation in the human serum containing cultures. All trypanosomes were completely lysed within 15, 20 and 30 minutes in the presence of 25% (V/V), 15% (V/V) and 5% (V/V) human serum, respectively (Fig. 8.1). Similarly in medium containing 25% (V/V) human serum the in vitro differentiated trypanosomes immediately began lysis and by approximately 15 minutes the lysis appeared to be complete. However trypanosomes in control cultures containing 25% fetal calf serum were very active and no trypanocidal activity was observed (Fig. 8.2).

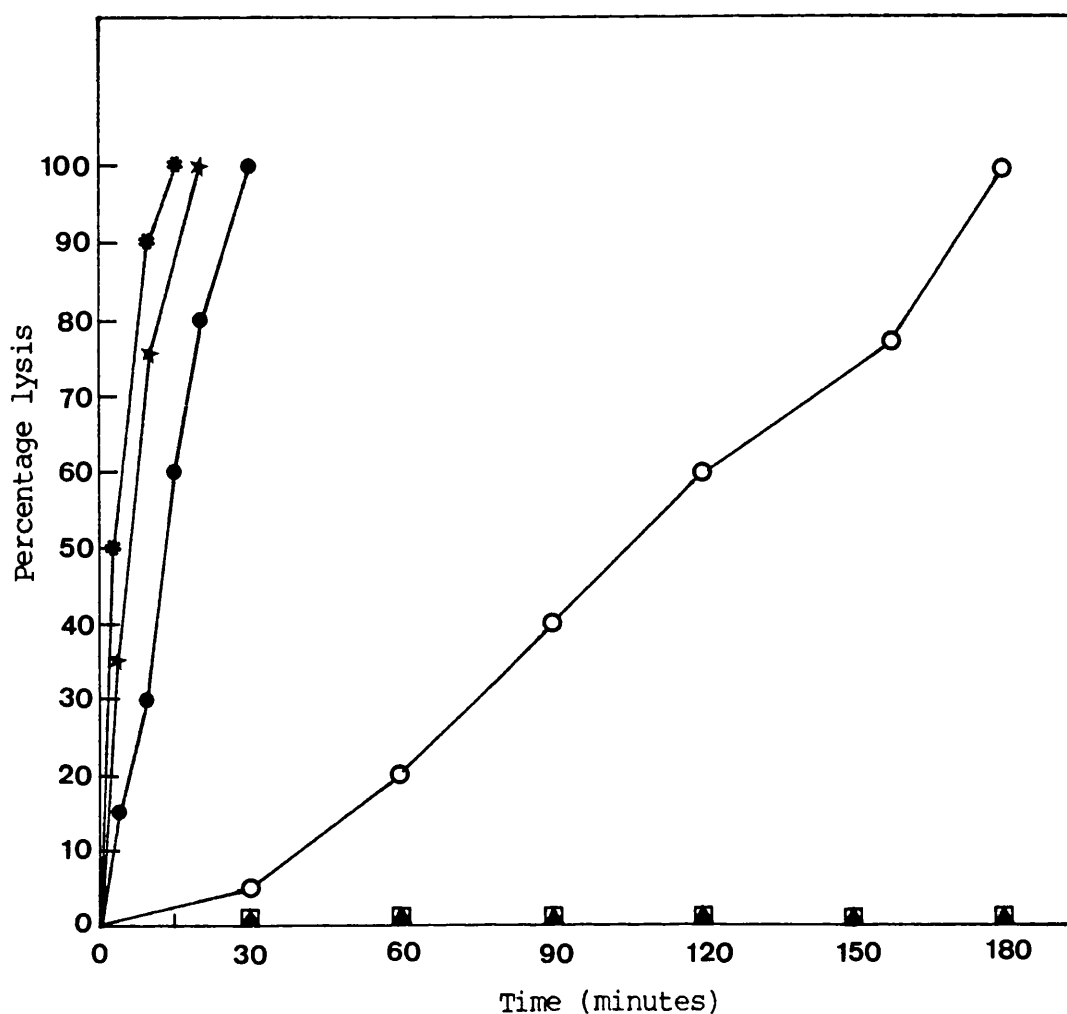


Fig. 8.1

Effect of unfractionated human serum on bloodstream and established procyclic trypanosomes. About 5×10^6 bloodstream trypanosomes were incubated, at 37°C , with either 25% (V/V) human (○) or 25% (V/V) rabbit serum (▲). Alternatively about 5×10^6 established procyclic trypanosomes were incubated, at 27°C , in medium containing 5% (●), 15% (★) and 25% (✱) human serum or 25% (V/V) fetal calf serum (□). The fraction of lysed trypanosomes were determined as described in the methods (section 8.2.2.2). Each point is the mean of 2 separate experiments in each of which trypanosomes were counted in duplicates.

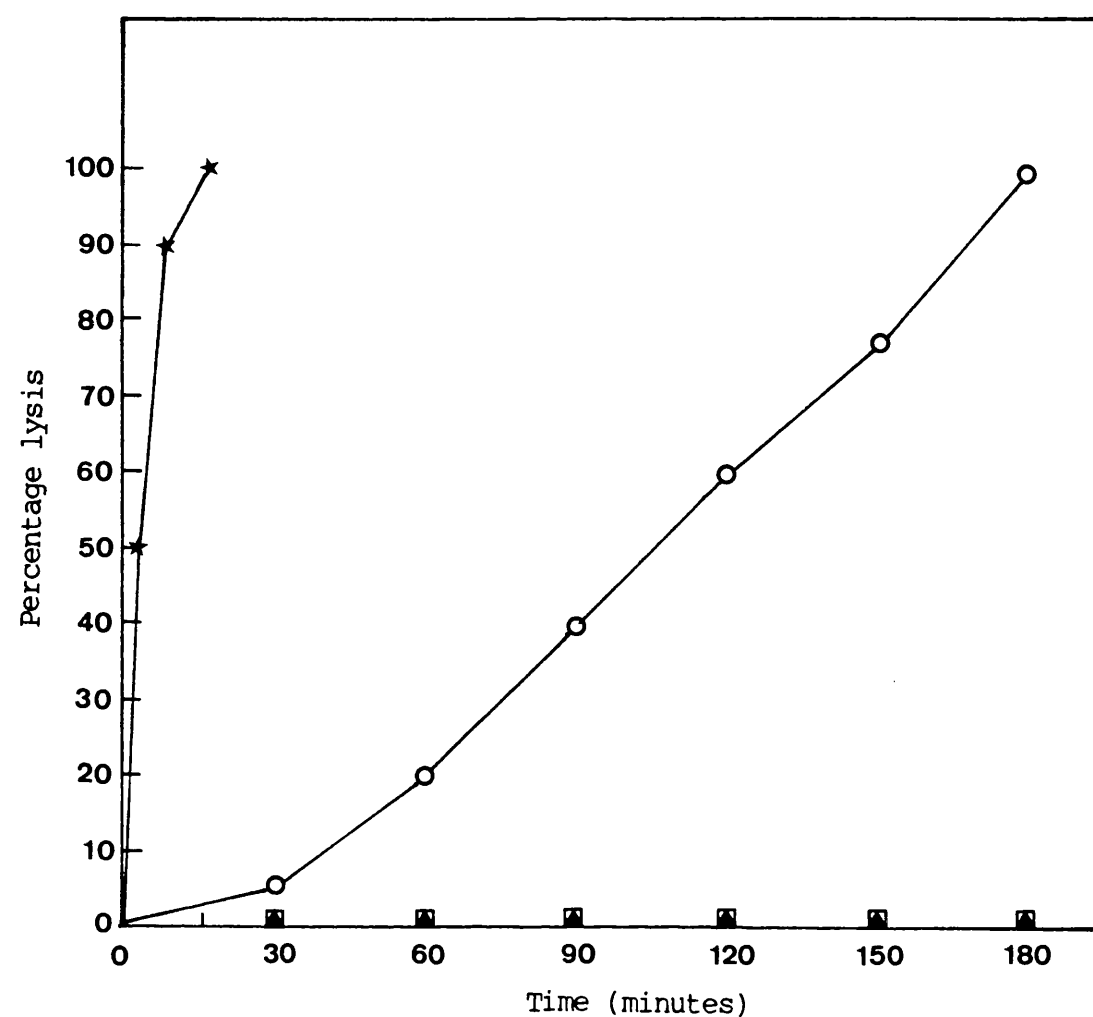


Fig. 8.2

Effect of unfractionated human serum on bloodstream and experimentally induced differentiation procyclic trypanosomes. About 5×10^6 bloodstream trypanosomes were incubated, at 37°C , with either 25% (V/V) human serum (○) or 25% (V/V) rabbit serum (▲). Alternatively about 5×10^6 experimentally induced differentiation procyclic trypanosomes were incubated, at 27°C , with either 25% (V/V) human serum (★) or 25% (V/V) fetal calf serum (□). The fraction of lysed trypanosomes were determined as described in the methods (section 8.2.2.2). Each point is the mean of 2 separate experiments in each of which trypanosomes were counted in duplicates.

8.4 Discussion

These results demonstrate that both established (isolated from the midgut of the fly and maintained in culture) and experimentally induced procyclic trypanosomes could be efficiently lysed by normal, non-immune, human serum. Although the bloodstream trypanosomes also did eventually lyse in the presence of 25% (V/V) human serum, this was a much slower process. This difference, most visible after approximately 15 minutes of incubation in 25% (V/V) human serum, could be utilised as a very effective and convenient tool for distinguishing between the bloodstream and procyclic forms of T.brucei. This difference in susceptibility to lysis is most likely due to the loss of the variable surface glycoprotein (VSG) coat of the bloodstream trypanosomes during both their in vivo and in vitro differentiation to the procyclic forms. The active ingredient of blood responsible for the lysis has previously been shown to be the high density lipoproteins (Rifkin, 1978).

CHAPTER NINE

CONCLUSIONS

9. Conclusions

Treatment of T.brucei with DNA damaging agents, DMS and γ -radiation increased the activity of ADPR-transferase in these organisms. Interestingly, the enzyme activity was found to be highest when the cellular NAD content was decreasing at the highest rate.

DMS a mono-functional methylating agent lowered the cellular NAD content in T.brucei in a dose dependent manner up to 300 μ M. However higher concentrations of DMS delayed the onset of NAD drop by some 70-90 minutes after exposure to the agent. The delayed onset of NAD reduction in cells treated with high concentrations of this DNA methylating agent could of course be due to either the inactivation of the enzyme itself and/or the direct methylation of the ADPR acceptor proteins, thus reducing their efficiency or capacity as acceptors of ADP-ribosylation. The cellular NAD level in T.brucei treated with any concentration of DMS used in this study (10 μ M to 1 mM) did not recover up to 3 hours of incubation at 37°C. The NAD drop caused by DMS treatment at any of the concentrations used was blocked by the ADPR-transferase competitive inhibitors 3AB and 3AAB. This was the case even though there is a considerable qualitative difference in the way that high or low concentrations of DMS affects the NAD levels in T.brucei. In the absence of DMS treatment the inhibitors on their own did not cause a drop, but instead a slight increase in the cellular NAD level.

γ -radiation also lowered the cellular NAD level to minimum in about 5 minutes in T.brucei in a dose dependent manner, however this recovered to near normal values within 15 minutes of treatment. This lowering of the NAD content in γ -irradiated mammalian cells is

usually associated with an even more rapid increase in the ADPR-transferase activity (Skidmore et al., 1979). The cellular NAD drop caused by γ -irradiation in T.brucei was blocked by 3AAB. Analysis of the effect of ADPR-transferase inhibitors on the nucleoid sedimentation rate of trypanosomes treated with either DMS or γ -radiation demonstrated the involvement of ADPR-transferase activity in the repair of both types of DNA damage. This is also evident in the enhancement of the cytotoxic effect of these two DNA damaging agents, by non-toxic concentration of 3AAB, on survival and growth of cultured trypanosomes.

There is no doubt that the ADPR-transferase inhibitors effectively slow or prevent the DMS and γ -radiation induced loss of NAD within cells. This confirms that the enzyme is indeed responsible for the DNA damage induced NAD loss.

In order to facilitate the study of ADPR-transferase during DNA repair and cellular differentiation an efficient in vitro culture system, permitting the continuous growth (see chapter 5) and differentiation (see chapter 6), of T.brucei was developed. Using this system the highest non-toxic concentration of ADPR-transferase inhibitors 3AB and 3AAB were found to be 2 mM and 200 μ M, respectively. The in vitro system also proved very useful for the study of effect of damaging agents on the trypanosomes in culture and the assessment of the effect of ADPR-transferase inhibitors on the recovery of the trypanosomes from DNA damage.

Study of the cytotoxic effect of DMS and γ -radiation on cultured T.brucei demonstrated that trypanosome survival and growth is very sensitive to these DNA damaging agents. However when trypanosomes were exposed to up to 15k rad of γ -radiation, the

cellular NAD level recovered to control levels within 15 minutes. This indicates that in γ -irradiated trypanosomes the cellular NAD level is not an important indicator of the potential ability of the trypanosomes to survive and recover from the DNA damage.

It has been shown that the competitive inhibitors of ADPR-transferase activity also inhibit the differentiation of chick myoblasts (Farzaneh et al., 1980 & 1982), the mitogen-induced activation of human peripheral blood lymphocytes (Johnstone & Williams, 1982), the induction of foetal enzymes in cultured hepatocytes (Althaus et al., 1982) and intracellular and extracellular differentiation of Trypanosoma cruzi (Williams, 1983b & 1984). In none of these studies did the inhibition of ADPR-transferase activity inhibit cell proliferation. Investigation of the effect of ADPR-transferase inhibitors on the survival and growth of cultured T.brucei also confirmed that these inhibitors, 3AB and 3AAB (at non-toxic concentrations), enhanced the cytotoxicity of DMS treatment and γ -irradiation. However they did not, at these concentrations, affect the cell growth and proliferation. Non-toxic concentrations of these inhibitors also blocked the experimentally induced differentiation of bloodstream forms of T.brucei to procyclic forms. These demonstrations provide further evidence suggesting that ADPR-transferase activity may be a general requirement for cellular differentiation but not proliferation.

The molecular basis for the requirement of ADPR-transferase activity in the repair of DNA strand-breaks is not fully understood. However it has already been established that ADPR-transferase activity is required for the efficient ligation of DNA strands during the excision repair of DNA. It is possible that the molecular reason for the requirement of ADPR-transferase activity during the repair of

DNA strand-breaks formed by exposure to DNA damaging agents (for review see Shall, 1984 & 1984a), or the repair of physiological DNA strand-breaks formed during cellular differentiation is the involvement of this enzyme in both processes of DNA strand-break ligation. The DNA strand-break formation has been reported in a number of examples of cellular differentiation (see section 1.5). Farzaneh et al., (1987) reported the transient formation of DNA strand-breaks during the induced myelocytic differentiation of the human promyelocytic leukaemic cell line, HL-60, in culture and that the subsequent ligation of these breaks requires nuclear ADPR-transferase activity. The activity of this enzyme was also reported to be required in morphological differentiation of Trypanosoma cruzi (Williams, 1983b & 1984) and the antigenic switching in Trypanosoma brucei (Cornelissen et al., 1985). Despite the fundamental importance of cellular differentiation, the understanding of the mechanisms controlling this process is very limited. DNA strand-break formation may be required for alterations in gene expression which is necessary for cellular differentiation.

In conclusion the evidence presented here demonstrates the involvement of ADPR-transferase activity in both the differentiation and the recovery of trypanosomes exposed to DNA damaging agents. Although the precise molecular reason for this involvement is not fully understood, the present weight of evidence suggests that it may be due to the requirement of this enzyme in the efficient ligation of DNA strand-breaks, be they formed by γ -irradiation, repair of the damaged bases after exposure to a methylating agent, or the physiological DNA strand-breaks formed during cellular differentiation (probably for regional chromatin relaxation and/or condensation).

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